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THE ROLE OF DLL4 / NOTCH SIGNALING IN LYMPHATIC DEVELOPMENT

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“The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!' (I found it!)
but 'That's funny ...''

Isaac Asimov (1920 - 1992)

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Aβ	Amyloid β
Abl	Abelson murine leukemia viral oncogene homolog
ADAM	a disintegrin and metalloproteinase
aISV	arterial intersomitic vessel
AMD	adrenomedullin
Ang	angiopoietin
AP	alkaline phosphatase
Aph-1	anterior pharynx-defective 1
APP	Amyloid precursor protein
Aspp1	Apoptosis-stimulating of p53 protein
BEC	blood endothelial cell
BM	bone marrow
BMP	Bone Morphogenetic Protein
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
ccbe1	collagen and calcium-binding EGF domain-1
CCL-21	chemokine (C-C motif) ligand 21 (=SLC)
CCR-7	C-C chemokine receptor type 7
CD	cluster of differentiation
cDNA	complementary DNA
CLEC-2	C-type lectin domain family 2
CMLC2	cardiac myosin light chain
COUP-TFII	chicken ovalbumine upstream promoter transcription factor II
COS	kidney cell line from African green monkey (CV-1 (simian) in Origin, and carrying the SV40 genetic material)
CSL	CBF1, Suppressor of Hairless (Su(H)), Lag-1
Ct	cycle treshold
DA	dorsal aorta
Dab2	Disabled homolog 2
DAPI	4',6-diamidino-2-phenylindole
DAPT	*N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DBM	DNA-binding mutant
DCLV	dorsal caudal lymph vessel
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DLAV	dorsal longitudinal anastomosing vessel
Dll	Delta-like
DLLV	dorsal longitudinal lymphatic vessel
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dPBS	Dulbecco's phosphate buffered saline
dpf	days post fertilization
DSL domain	Delta:Serrate:Lag-2 domain
DsRed	Discosoma sp. red fluorescent protein
E	embryonic day
EC	endothelial cell
ECD	extracellular domain
EDTA	ethylenediaminetetraacetic acid
Efnb2	EphrinB2

EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EGM2	endothelial cell growth medium
EphB4	Ephrin type-B receptor 4
ENU	*N*-ethyl-*N*-nitrosourea
EPC	endothelial progenitor cell
ERK	extracellular-signal-regulated kinase
FACS	fluorescence-associated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable region (tail region of an antibody)
FCS	fetal calf serum
FGF(R)	fibroblast growth factor (receptor)
Fli1	Friend leukemia virus integration 1 (transcription factor of the ETS family)
Flt / Flk	FMS-like tyrosine kinase
Foxc	Forkhead transcription factor
GFP	green fluorescent protein
Gal4	yeast galactose-induced transcription activation protein 4 (binds UAS DNA-domain)
GM-CSF	granulocyt-macrophage colony stimulating factor
grl	gridlock (zebrafish homolog of Hey2)
GSK3β	Glycogen synthase kinase 3 β
Hes	Hairy and Enhancer of Split
Her	Hairy and Enhancer of Split – related
Hey	Hairy/enhancer-of-split related with YRPW motif
Hh	Hedgehog
HMVEC-DLy	human microvascular endothelial cell – dermal lymphatic
HMVEC-LLy	human microvascular endothelial cell – lung lymphatic
hpf	hours post fertilization
HSC	hematopoietic stem cell
HSP70	heat shock protein 70
HUVEC	human umbilical vein endothelical cell
Ig	immunoglobulin
I-SceI	homing endonuclease from *Saccharomyces cerevisiae*
ISV	intersomitic vessel
KD	knockdown
kdr	Kinase insert domain receptor (=VEGFR2)
kdr-l	kdr-like
KSHV	Kaposi sarcoma herpes virus
LEC	lymphatic endothelial cell
LEF	Lymphoid enhancer-binding factor
LISV	lymphatic intersomitic vessel
LISV-PL	parachordal lymphangioblasts that form the LISV
LPM	lateral plate mesoderm
Lyve-1	lymphatic vessel endothelial hyaluronan receptor 1
MAB(T)	maleic acid buffer (supplemented with Tween-20)
MAPK	mitogen-activated protein kinase
mib	mindbomb
MMR	Marc's Modified Ringer's medium
MO	morpholino
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MyoD	Myogenic Differentiation 1
NECD	Notch extracellular domain
Neur	Neuralized
NICD	Notch intracellular domain

NLS	nuclear localization signal
No	notochord
Nrarp	Notch-regulated ankyrin repeat protein
Nrp	neuropilin
NT	neural tube
OL	oligodendrocyte
OPC	oligodendrocyte precursor cell
PAV	parachordal vessel
PBS(T)	phosphate buffered saline (supplemented with Tween-20)
PCV	posterior cardinal vein
PDGF-B	platelet-derived growth factor subunit B
PDGFR-β	β-type platelet-derived growth factor receptor
Pdpn	podoplanin
PDZ domain	common structural domain in signaling proteins (PDZ = post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1))
PECAM-1	platelet endothelial cell adhesion molecule 1 (=CD31)
Pen-2	presenilin enhancer 2
PEST	proline (P) – glutamate (E) – serine (S) – threonine (T)
PI3K	phosphatidylinositol 3-kinase
PL	parochordal lymphangioblast
Plcg	Phosphoinositide phospholipase C gamma
Prox1	Prospero homeobox protein 1
PS	presenilin
PTU	phenylthiourea
RBP / RbpJ	recombination signal binding protein of the Jκ immunoglobulin gene (= Su(H) / CBF-1 / CSL)
REMI	restriction enzyme mediated integration
RFP	red fluorescent protein
Rictor	rapamycin independent companion of mTOR
RLT	RNeasy lysis buffer (Qiagen)
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean
Shh	Sonic hedgehog
SLC	Secondary lymphoid tissue chemokine (=CCL-21)
SLP76	SH2 (SRC (sarcoma) homology 2) domain containing leukocyte protein of 76kDa
SMC	smooth muscle cell
SOX	SRY(sex determining region Y) -related HMG (High Mobility Group)-box
SPP	Signal Peptide Peptidase
SSC(T)	saline-sodium citrate buffer (supplemented with Tween-20)
Su(H)	Suppressor of Hairless
SYK	spleen tyrosine kinase
TACE	tumor necrosis factor-α-converting enzyme (=ADAM17)
TBX20	T-box 20 (homeobox transcription factor)
TCF	T-cell factor
TD	thoracic duct
TGF-β	transforming growth factor β
TNF	tumor necrosis factor
TRITC	tetramethyl rhodamine isothiocyanate
UAS	upstream activation sequence
VCLV	ventral caudal lymph vessel

VE-cadherin	vascular endothelial cadherin
VEGF(R)	vascular endothelial growth factor (receptor)
vISV	venous intersomitic vessel
Wnt	Wingless (*Drosophila* gene) – Integration (mammalian homologous gene)
YFP	yellow fluorescent protein
ZFIN	Zebrafish Information Network
ZFN	Zinc finger nuclease
ZIRC	Zebrafish International Resource Center

PREFACE

Despite the importance of lymph vessels in both health and disease, very little is known about the genetic regulation of lymphangiogenesis, the formation of lymph vessels. The Notch signaling pathway is involved in many developmental processes. During angiogenesis, the formation of blood vessels, Notch regulates arterial *versus* venous differentiation of blood endothelial cells and controls the branching process of the blood vessel network. The molecular mechanisms determining the establishment of lymphatic endothelial cells, the third type of endothelial cells, and the outgrowth of an elaborate network of lymph vessels, are still poorly understood. Several indications suggest a role for Notch in all three EC types. Components of the Notch pathway are expressed in LECs and several Notch-related genes that are important for arterial differentiation are also required in lymphatic development. We therefore reasoned that Notch signaling itself might also be involved in lymphangiogenesis. In this study we investigated the role of the Notch signaling pathway in lymphangiogenesis using zebrafish embryos and tadpoles, two small animal models that allow high-throughput screening for lymphatic genes. By selecting chemical and genetic silencing strategies to avoid vascular defects, we now identified for the first time a dual role for Notch signaling in developmental lymphangiogenesis.

Chapter I

INTRODUCTION

1 LYMPHANGIOGENESIS

1.1 THE LYMPHATIC SYSTEM

The lymphatic system has long remained in the shadow of the blood vascular system. This 'second' circulation system was merely looked at as a drainage network. About one decade ago, the lymphatic circulation gained more and more interest of scientists due to its importance as a key player in several diseases and its crucial role in normal functioning of the body of vertebrate organisms.

1.1.1 Structure

The lymphatic system is organized as an elaborate network of vessels that end blindly in capillaries, throughout the entire body except the central nervous system and avascular tissues like epidermis and cartilage. These capillaries have a unique structure that allows the uptake of fluids from the surrounding tissue, but prevents it from leaking out. The endothelial cells that make up the thin wall of the lymphatic capillaries are slightly overlapping and loosely connected to each other. When fluid pressure in the surrounding tissue is higher than in the lumen of the lymph vessel, the cells are pushed apart and open up like a one-way swinging door. However, when pressure inside the vessel is higher than outside, the cell junctions are tightly closed and fluid is prevented from leaking out. The thin-walled capillaries are prevented from being collapsed under this pressure by the attachment of anchoring filaments that secure the lymphatic endothelial cells (LECs) to the surrounding matrix and tissue [1,2]. Furthermore, under high fluid pressure the anchoring filaments are stretched making the gaps between the endothelial cells even larger and thus allowing faster uptake of fluid [3]. Baluk et al [4] have made high-resolution images of the junctions of endothelial cells in lymphatic capillaries. They found that these cells have oak leaf-like shapes with overlapping flaps that lack junctions at the tip but are anchored on the sides by discontinuous button-like junctions that differ from the conventional, continuous zipper-like junctions in collecting lymphatics and blood vessels [4] (Figure 1A-C). This organization allows fluids to enter the capillaries via openings between the buttons, without disrupting the junctional integrity between the lymphatic endothelial cells (Figure 1D).

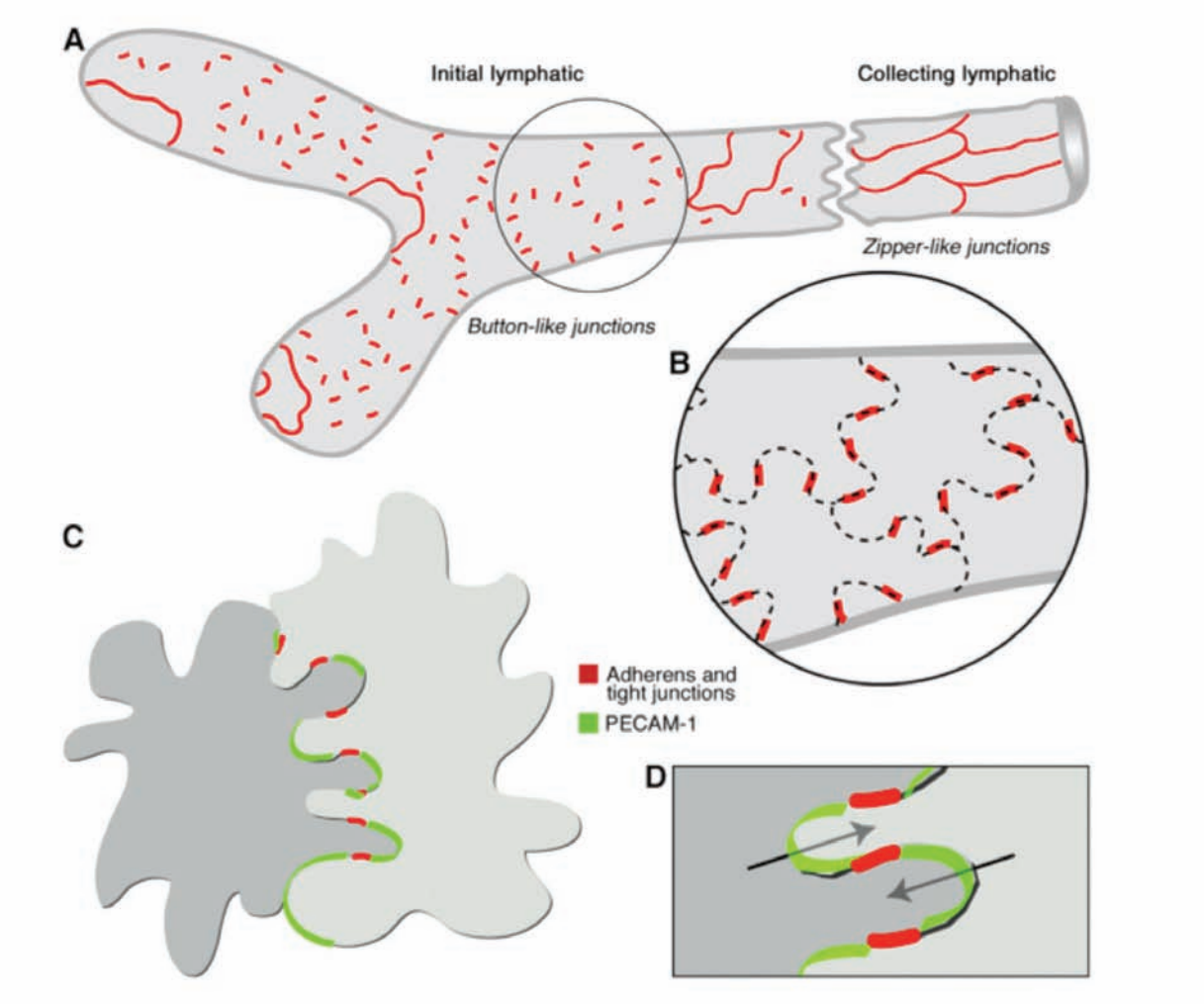

Figure1: *Oak-leaf like lymphatic endothelial cells with button-like junctions in lymphatic capillaries. (A) Schematic drawing of the endothelium of an initial lymphatic (capillary) with discontinuous buttons and a collecting lymphatic with continuous zipper junctions. (B) Oak leaf shape of endothelial cells in lymphatic capillaries. Buttons with adherens and tight junctions are indicated in red. (C-D) Most PECAM-1 expression is at the tips of flaps. Neighbouring LECs have overlapping edges (C). Fluid is taken up through the loose flaps (D).* [4]

After being taken up by the capillaries the lymph fluid is transported by collecting lymph vessels. These vessels are lined by LECs and have a thin layer of smooth muscle cells. Valves ensure that the lymph can only be transported in one direction. The lymph vessels transport the lymph to either the right lymphatic duct or the thoracic duct, the largest

lymph vessel in the body. These vessels eventually drain the lymph into the right and left subclavian veins respectively and in this way return it to the general circulation.

Comparative phylogenetic analysis has shown that a true lymphatic system is present only in vertebrates [5]. Primitive fish have a lymphatic-like secondary vascular system, but this contains blood and is considered to be part of the circulatory system. Teleost fish, like zebrafish and Medaka, are probably the first vertebrates with a lymphatic system that is anatomically distinct from the blood vasculature ([6,7] and Tomonori Deguchi, personal communication).

1.1.2 Functions in health and disease

1.1.2.1 normal functions

As explained above, the primary function of the lymphatic system is the reabsorption of interstitial fluids that have leaked out from the blood vessels. Due to the action of oncotic pressure, interstitial fluid leaks out from the blood vessels at the arterial side of the capillary bed where pressure is higher in the blood vessels than in the surrounding tissues. The interstitial fluid forms the immediate surrounding of the cells to which they constantly add and remove substances. Most of the interstitial fluid is taken up again by the venules. The remaining 10-20% is taken up by the lymphatics and returned to the circulation as lymph [8,9].

Secondly, lymphatics in the gastrointestinal tract, predominantly the small intestine, are responsible for the uptake of lipids from the digestive tract. While most other nutrients are taken up directly into the blood stream and are transported through the portal vein to the liver, lipids are first taken up by lymphatics to be subsequently transported to the blood circulation. These intestinal lymphatics are called lacteals and the milky white lymph they transport, containing emulsified fat, is called chyle [10].

Finally, the lymphatic network is of critical importance for the normal function of the immune system. Lymphatics pass through several lymphoid organs, especially lymph nodes where antigen presenting cells and foreign substances that were taken up from the interstitial fluid are removed from the lymph and lymphocytes are added [10].

1.1.2.2 lymphedema

Malfunctioning of the lymphatic system causes accumulation of interstitial fluid in the tissues, which leads to swelling or lymphedema. This can lead to quite severe and disfiguring effects usually at the limbs, but also face, neck and abdomen can be affected. When malfunctioning is caused by damage to the lymphatics, for example after surgery or infection with certain parasites, it is called acquired or secondary lymphedema. Congenital or primary lymphedema is caused by genetic mutations and is less common [10]. Furthermore, when lymphedema is caused by a (temporary) increase in the production of waste material (leading to increased osmotic pressure in the tissues) it is called dynamic edema. Static edema in contrast, is caused by damage to the lymphatic system and does not disappear by itself [11]. Today, the most important treatment still is manipulation through massage and bandaging. So far, no surgical or genetic treatment is being applied on lymphedema patients [11].

1.1.2.3 cancer metastasis

Metastasis, the spread of tumor cells from the primary tumor to different parts of the body, is thought to be responsible directly or indirectly for more than 90% of all cancer deaths [12]. Many tumor types, including melanoma, breast and prostate cancers first metastasize to their regional lymph nodes via lymph vessels, after which they are disseminated to other parts of the body. Therefore, the presence of metastatic tumor cells in the regional lymph nodes of a tumor is an indication of poor prognoses and is used for determination of tumor stage.

Only recently it was shown that the lymphatic system plays an active role in cancer metastasis via direct interaction between tumors and lymph vessels. First of all, their architecture makes lymphatics more amenable to the entry of invasive tumor cells than blood vessels. They have loose overlapping cell-cell junctions and no or only an incomplete basement membrane. Furthermore, malignant tumors are often associated with an increased density of lymph vessels, mostly in the peritumoral region. Intratumoral lymphatics are rare and are considered to be nonfunctional due to mechanical compression [13]. This increased density is caused by co-option of existing lymphatics by the tumor and by

induction of the outgrowth of new lymph vessels. The induction of lymphangiogenesis, i.e. the formation of lymph vessels, by tumors is mediated by prolymphangiogenic factors and cytokines that can be produced by the tumor cells themselves, or by stromal cells, tumor-associated macrophages or damaged platelets [14-16]. In addition to local lymphangiogenesis, tumor-derived vascular endothelial growth factor (VEGF)-A and VEGF-C can also induce lymphangiogenesis within the tumor-draining lymph nodes, thereby promoting the flow of lymph to them and increasing the efficacy of transport of disseminating tumor cells to the lymph nodes [17,18]. On the other hand, lymphatics also communicate to tumors. LECs have been shown to produce the chemokine CCL-21. Tumor cells that express the cognate receptor CCR-7, chemotactically migrate towards these CCL-21 producing LECs, thereby promoting the entry of tumor cells into the lymphatics [19,20].

1.1.2.4 inflammation

Inflammation is a local or systemic reaction of the immune system caused by external or internal stimuli, e.g. microbial infections or malignantly transformed cancer cells, with the objective to remove the source of the injury, inhibit its further dissemination and eventually repair damaged tissue. However, when this process is not controlled and inflammation spreads, it loses its repairing function and may even cause damage. Blood vessels play an important role in the inflammatory process and inflamed tissue is characterized by hyperaemia and increased permeability of capillaries. Recent studies provide evidence that lymphatic vessels actively participate in acute and chronic inflammation as well and that they are not just a passive route for circulating lymphocytes. During inflammation lymphangiogenesis is induced, mainly to facilitate removal of the accumulated interstitial fluid because of increased vascular permeability induced by angiogenic and inflammatory stimuli.

Proinflammatory cytokines produced by inflammatory cells induce VEGF-C expression in fibroblasts, stimulating lymphatic vessel growth at inflammation sites [21]. Also macrophages that are attracted to inflammation sites can transform into VEGF-C/D producing cells to stimulate the growth of lymphatic vessels [15]. Furthermore, macrophages are suggested to have a dual role in inflammation-induced lymphangiogenesis since they

can also contribute to lymphangiogenesis by transdifferentiating into LECs, which can incorporate into the lymphatic endothelium [22,23].

Many inflammatory diseases seem to be associated with lymphatic activation and dysfunction. Kidney transplant rejection is often accompanied by massive lymphatic neoangiogenesis, induced by macrophages producing VEGF-C. These new lymph vessels produce CCL-21 (ligand of chemokine receptor CCR-7), which attracts CCR-7^{+} lymphocytes and dendritic cells and might actively promote the inflammatory process [24]. Similar examples of inflammatory diseases associated with lymphatic hyperplasia are psoriasis, chronic airway inflammation or astma and rheumatoid arthritis [25-27].

1.2 LYMPHATIC DEVELOPMENT IN MAMMALS

1.2.1 History

Despite only recent advances in the genetic basis of lymphatic development, the existence of the lymphatic system has been known since antiquity. Already in 400 BC Hippocrates (460 – 377 BC) observed vessels containing 'white blood' when studying the axillary lymph nodes but only in 1627 Gasparo Aselli (1581 – 1626) described the lacteal vessels as 'milky veins' in a 'well-fed' dog [28]. In the early 20th century, vascular anatomists such as Florence Sabin (1871 – 1953) characterized the anatomy of the lymphatic system in more detail. By using dye injections and histological methods they found that the lymphatic system is an extensive network that is ramified throughout the entire body [29,30]. At that time two competing theories were proposed about the origin of lymphatics. Based on her studies Florence Sabin proposed that primitive lymph sacs originate from endothelial cells that bud from the embryonic veins during early development [29,30]. In contrast, the theory put forward by Huntington (1861 – 1927) and McClure (1852 – 1944) describes that rudimentary lymphatics first arise independently in the mesenchyme and only later establish connections with the venous system [31]. For almost a century, this controversy has remained and it was only a few decades ago that new insights were gained into the genesis of the lymphatic system. This delay was largely due to the lack of reliable molecular markers for

the detection of LECs and to the lack of identified regulators of lymphangiogenesis. However, several markers have been recently identified that have allowed significant advances in the understanding of lymphatic development. These new findings have largely confirmed Sabin's original theory of mammalian lymphatic development, although in birds and frogs recruitment of lymphangioblasts also contributes to the formation of lymphatics [32-34]. Studies in mice have provided significant evidence for the origin of the mammalian lymphatic system from embryonic veins [35]. Of the known lymphatic-specific markers, several only acquire their specificity as embryonic development progresses, so differentiation of the lymphatic vasculature is probably a stepwise process. For example, the expression pattern of vascular endothelial growth factor receptor 3 (VEGFR3) during embryonic development has provided additional support for Sabin's model. VEGFR3 was the first lymphatic-specific growth factor receptor identified [36], but is initially expressed in angioblasts of murine head mesenchyme, dorsal aorta, cardinal vein and allantois [36,37]. At embryonic day 12.5 (E12.5), VEGFR3 is expressed both in developing venous and in presumptive lymphatic endothelia, and only later, in adult tissues, VEGFR3 is largely restricted to the lymphatic endothelium [36,38]. These findings suggested that VEGFR3 plays a role in the development of both the vascular and lymphatic system. This was confirmed by the fact that *VEGFR3* null mice are not viable and die from cardiovascular failure even before the emergence of the lymphatic vessels [37]. More direct evidence for the venous origin of lymphatics came from a Cre/loxP-based lineage-tracing study using Prox1-promoter elements, that showed that, at least in mammals, the lymphatic vasculature is derived from venous ECs and that hematopoietic cells do not contribute to the developing lymph sacs [35]. Finally, the overlap of several other molecular markers between LECs and blood endothelial cells (BECs) demonstrates the close structural and developmental relationship between the blood and lymphatic systems. The current model of early lymphatic development describes that the expression of a limited number of additional genes by certain BECs induces the step-wise process of lymphatic competence, commitment, differentiation and maturation that ultimately leads to the formation of the lymphatic network (Figure 2).

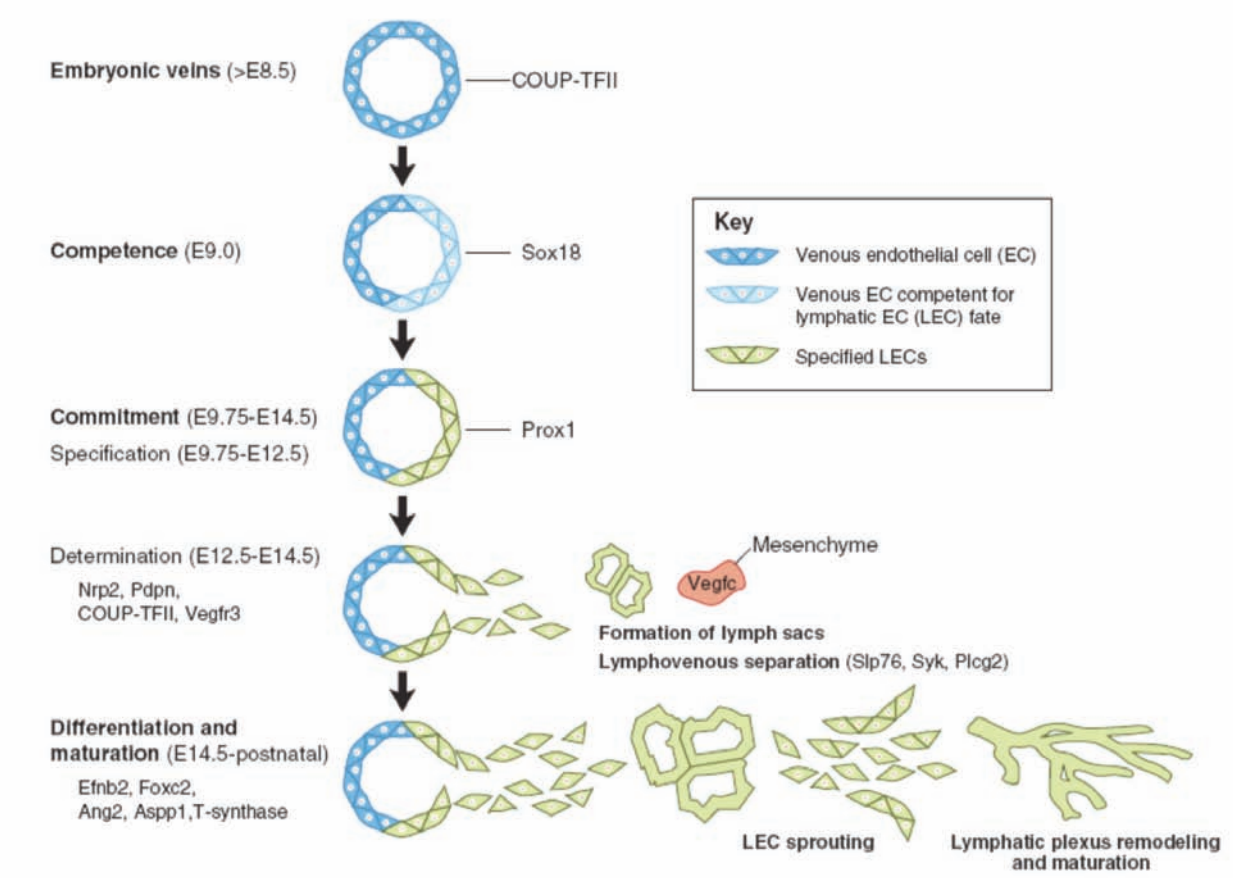

***Figure 2**: Model of lymphatic development (Oliver & Srinivasan, 2010 [39]). For details: see text.*

1.2.2 Competence, commitment and differentiation of LECs

Lymphatic development in the mouse starts at about E9-9.5 when venous endothelial cells become competent to respond to a lymphatic-inducing signal. Competence denotes the ability of cells to respond to an inducing signal [39]. The molecular factor(s) that regulate the initial stage of lymphatic competence remain unknown. However, the expression of the lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) in the cardinal veins can be considered as an indication that venous cells are competent [40] (Figure 2). Despite this early expression of Lyve-1 in lymphatic progenitor cells, *Lyve-1* null mice develop a largely normal lymphatic vasculature, arguing against a role of Lyve-1 in establishing LEC identity

[41,42]. Specific expression of *Prox1* at E9.5 in a subpopulation of these Lyve-1 positive cells is the first indication that lymphangiogenesis has begun. This step is called 'lymphatic commitment' which denotes that the developmental potential of these cells becomes restricted [39]. At about E10.5 these Prox1 positive cells, which are located on one side of the cardinal vein, start to bud off in a polarized manner (Figure 2). After budding these cells start expressing higher levels of additional lymphatic endothelial markers such as Secondary Lymphoid Tissue (SLC) chemokine and VEGFR3. Expression of VEGFR3 in BECs decreases. The expression of Prox1, Lyve-1, SLC and VEGFR3 probably indicates that the cells are irreversibly committed to the lymphatic pathway (Figure 2). The commitment stage is characterized by an initial labile phase of specification (when a cell is able to differentiate autonomously if placed in a neutral environment) and a second stage of determination (when committed cells will differentiate autonomously even if placed into another tissue context) [39,43].

Prox1 has been described as the key transcription factor determining lymphatic development. Prox1 is a homeobox transcription factor that is homologous to the Drosophila *prospero* gene. Analysis of its expression pattern suggested that it has a functional role in a variety of tissues, including lens, heart, liver, pancreas and the central nervous system [44]. Later, Wigle and Oliver proved that Prox1 is a specific marker of a subpopulation of ECs in the cardinal vein that by budding and sprouting give rise to the lymphatic system [45]. This finding thus fully validated Sabin's proposal of the venous origin of the primary lymph sacs [29,30]. Analysis of *Prox1* null embryos showed that early in development the initial budding of the ECs that give rise to the lymphatic system appeared to be unaffected and is thus Prox1 independent. However, maintenance of endothelial budding and sprouting is Prox1 dependent as these processes were completely blocked in Prox1 null mice, causing the arrest of lymphatic development. Vasculogenesis and angiogenesis are unaffected in these mice [45]. These results demonstrate that Prox1 activity is essential for normal lymphatic development and that vascular and lymphatic development are independent processes. More recently, different groups have shown that Prox1 expression is not only necessary, but also sufficient to induce transdifferentiation of BECs to LECs [46,47]. Adenoviral overexpression of Prox1 in BECs results in induction of LEC-specific gene transcription and suppresses the expression of ±40% of BEC-specific genes, thus

identifying Prox1 as a master switch in the lymphatic differentiation process [46,47]. Once more, this indicates that during evolution the lymphatic vasculature originated from the blood vasculature by the additional expression of only a few gene products such as Prox1. Finally, it has also been shown that conditional downregulation of *Prox1* during embryonic, postnatal or adult stages is sufficient to reprogram LECs into BECs [48]. Therefore, Prox1 appears to be a binary switch that suppresses BEC identity and promotes and maintains LEC identity. However, this interchangeability of BEC and LEC identity is remarkable and raises questions as to how specified endothelial cells really are, since differentiation or specification is considered to be irreversible.

Defects in the transcription factor *SOX18* (SRY (sex determining region Y) box 18) cause lymphatic dysfunction in the human syndrome hypotrichosis-lymphoedema-telangiectasia [49]. It has been shown that *SOX18* is expressed in a subset of cardinal vein cells that later co-express *Prox1* and bud off to form the primary lymph sacs (Figure 2). Overexpression of *SOX18* in BECs induces them to express *Prox1* and other LEC-specific markers. *SOX18* null embryos fail to express *Prox1* in the cardinal vein and develop edema due to a complete blockade of LEC differentiation from the cardinal vein [50]. Further investigation determined that SOX18 directly controls *Prox1* expression by binding to its promoter [50]. Altogether these findings demonstrate a critical role for SOX18 in lymphatic development even upstream of Prox1. It is not certain whether SOX18 rather than Prox1 is the actual key transcription factor regulating lymphatic differentiation or whether SOX18 expression merely makes ECs competent. In contrast to *Prox1*, *SOX18* expression is only detected up to ±E14.5 [50]. The loss of *SOX18* expression after this stage indicates that SOX18 is only required during the early stages of lymphatic development, perhaps solely to induce *Prox1*, which is also required for maintenance of the lymphatic identity at later stages [48].

1.2.3 Maturation and wiring of the lymphatic network

Once LECs have been specified from the cardinal vein, they migrate and proliferate to establish the initial lymph sacs and early lymphatic vessels. VEGFR3 controls both proliferation and migration of LECs [51]. Although *VEGFR3* is initially expressed in both blood

and lymphatic vessels, its expression becomes restricted to LECs at E12.5 [36]. In humans, mutations in the *VEGFR3* gene cause Milroy Disease, a form of congenital lymphoedema caused by hypoplasia of the lymphatic vessels [52]. The ligands for VEGFR3 are VEGF-C and VEGF-D [53,54]. VEGF-C is expressed by vascular smooth muscle cells and mesenchymal cells close to the migratory destination of lymphatic progenitors [55,56]. VEGF-D is produced in a dynamic pattern most strongly in the lung, kidney and mesenchymal cells in the skin [53]. Further, also myeloid cells have been identified as sources of VEGF-C and –D [25,57,58]. Retroviral or transgenic overexpression of either VEGFR3 ligand in mice is sufficient to induce lymphangiogenesis in the areas of misexpression [59-61]. *VEGF-C* null mice show normal specification of LECs, but absence of LEC budding and formation of primary lymph sacs due to the absence of the source of VEGF-C in the mesenchyme [55]. In contrast, *VEGF-D* knockout mice have no discernible lymphatic defect, suggesting that it does not play a crucial role in lymphatic development [62]. Neuropilin2 (Nrp2) is a nonsignaling transmembrane receptor that acts as a co-receptor for VEGFR3 and is expressed in veins and lymphatic vessels [63]. *Nrp2* null mice show absence of small lymphatic vessels and capillaries during development, but blood vessels and larger collecting lymphatic vessels develop normally, suggesting that Nrp2 is selectively required for the formation of small lymphatic vessels and capillaries [63]. Recently it has been shown that interaction between Nrp2 and VEGFR3 mediates lymphatic tip cell extension and guided sprouting of lymphatic vessels in response to VEGF-C [64]. In addition to VEGF-C and VEGF-D, adrenomedullin (AMD) signaling is required for normal LEC proliferation and disruption of AMD signaling results in edema and embryonic lethality. LEC specification occurs normally but lymph sacs are hypoplastic and lined with thin LECs [65].

The initial lymphatic vascular plexus undergoes extensive expansion and remodeling that goes on postnatally. Various genes have been shown to play important roles during this remodeling. Mutations in the forkhead transcription factor 2 (FOXC2) cause lymphedema-distichiasis, an autosomal dominant disorder characterized by lymphedema with onset around puberty and a double row of eyelashes (distichiasis) [66]. *FOXC2* is expressed in lymphatic progenitors budding from the cardinal vein, jugular lymph sacs, lymphatic collectors and capillaries, as well as in podocytes and developing eyelids [66]. *Foxc2*$^{-/-}$ mice die embryonically after E12.5 and demonstrate cardiac and skeletal abnormalities. *Foxc2*$^{+/-}$

mice develop generally normal but have characteristics reminiscent of lymphedema-distichiasis in humans, such as distichiasis and hindlimb lymphedema [67]. The lymphedema phenotype is caused by an abnormal pattern of the lymphatic vessels, increased pericyte investment, agenesis of lymphatic valves and lymphatic dysfunction [68]. So, FOXC2 is essential for the morphogenesis of lymphatic valves and the establishment of pericyte-free lymphatic capillaries. In the latter process FOXC2 cooperates with VEGFR3 [68,69]. Compound *Foxc1*$^{+/-}$*;Foxc2*$^{-/-}$ embryos show reduced numbers of Prox1 positive cells budding from the cardinal vein and abnormal formation of lymph sacs [69]. This indicates that the FOXC transcription factors are also important for the initial sprouting of LECs from the cardinal vein. Another important mediator, necessary for the remodeling and maturation of the lymphatic network, is EphrinB2 [70]. More precisely, the PDZ interaction site of EphrinB2 appears to be crucial, since mice homozygous for a deletion of the PDZ interaction site (*ephrinB2*$^{\Delta V/\Delta V}$) exhibit major lymphatic defects. These mice have normal blood vascular remodeling, but fail to remodel the primary lymphatic capillary plexus into a hierarchical vessel network, have hyperplasia, and lack luminal valves [70]. The PDZ domain effectors that are required to mediate the lymphatic function of EphrinB2 are not yet known.

Lymphatic vessels return lymph fluid to the blood, but both vascular systems connect only at the left and right subclavian vein. In the remainder of the body it is critical that both systems are close together but remain separated. The correct separation of blood and lymphatic vessels is mediated by the adaptor protein SLP-76 (SRC homology 2 – domain containing leukocyte protein of 76 kDa) and the tyrosine kinase SYK (Spleen Tyrosine Kinase) (Figure 2). Both molecules are mainly expressed by hematopoietic cells. Mutant mice show abnormal shunting between lymphatics and blood vessels resulting in blood filled lymphatics [71,72]. How SLP76- and SYK-signaling pathways in hematopoietic cells mediate this function has remained unknown for a long time. Very recently however, a link has been made with the role that was described for podoplanin in the developmental separation of the blood and lymphatic circulation [73]. Interaction of endothelial podoplanin in the developing lymph sacs with circulating platelets form the cardinal vein induces platelet activation and aggregation. These platelet aggregates are thus built up at the separation zone of podoplanin positive lymph sacs and cardinal veins, thereby closing the junction between them [73]. Lack of SYK or SLP-76 affects the capacity of platelets to activate

integrins, and to undergo aggregation and activation [74]. Therefore, the formation of the separating platelet plug is compromised in mutants with deficiencies of these proteins. A similar mutant phenotype of blood-filled lymphatics was observed in *Plcg2* (Phospholipase Cγ2) mutant mice [75]. Also this phenotype can be brought back to deficient platelet aggregation as Plcγ2 is phosphorylated by SYK and SLP-76 [71,74]. SYK, SLP-76 and Plcγ2 act downstream of CLEC-2, a podoplanin-specific receptor on platelets that is responsible for platelet aggregation induced by podoplanin [76,77]. However, SYK deficiency causes much faster and more severe blood-lymphatic abnormalities compared to those seen in *podoplanin* null mice [78,79]. SYK-expression was additionally identified in myeloid cells that express VEGF-C and –D and that show strong lymphangiogenic activity upon transplantation into the skin [80]. Loss of SYK results in elevated VEGF-C/-D production and lymphatic hyperplasia, which leads to direct contact of BECs and LECs and ultimately to the formation of blood-lymphatic junctions [80].

1.3 LYMPHATIC DEVELOPMENT IN ZEBRAFISH

1.3.1 Zebrafish as a small animal model

Zebrafish, or *Danio rerio*, is a small (adults 3-5 cm) tropical freshwater fish belonging to the *Cyprinidae* family. It is an important vertebrate model organism in scientific research for studies on vertebrate development and gene function. It has been used in many fields including developmental biology, oncology, toxicology, genetics, neurobiology, etc because of its numerous advantages for use in the lab as a model system. Zebrafish have a very rapid generation, they grow from embryo to free-living animal in less than 5 days, and within 36 hours post fertilization (hpf) precursors to all major organs have been established. Because of the transparency of the embryos, development of multiple organ systems and tissues can be easily viewed and analyzed. At about 3 months of age the fish are fertile, with females being able to spawn hundreds of eggs every week all year long. This high fecundity and rapid generation makes zebrafish an ideal model organism for high-throughput screenings of chemical compounds or gene knockdown (see below). Moreover,

its genetic code is almost completely sequenced and annotated (www.sanger.ac.uk/Projects/D_rerio) and several well-characterized mutants have been established and are commercially available (Znomics, www.znomics.com). Also other resources like transgenic fish lines, antibodies, protocols, etc have been collected in an online biological database and made publicly available (Zebrafish Information Network (ZFIN, zfin.org) and Zebrafish International Resource Center (ZIRC, zebrafish.org)). Genetic manipulation of zebrafish embryos is relatively easy. By injecting morpholino antisense oligomers in the 1-4 cell stage, genes can be knocked down for up to 4 days by blocking translation of mRNA (ATG-targeting morpholino) or splicing of pre-mRNA (splice-site targeting morpholino) [81]. Several techniques exist to generate stable transgenic zebrafish lines like retroviral transduction, recombination-mediated insertion via a transposase (e.g. Tol2) or integrase system (e.g. ϕC31), restriction-enzyme mediated insertion (e.g. I-SceI meganuclease), etc. Currently, the most frequently used technique in zebrafish is the transposon-based integration with the Tol2 transposable element of the medaka fish *Oryzias latipes* [82]. The gene of interest is cloned into the Tol2 transposon element and the DNA vector containing this construct is then injected into 1-cell stage embryos together with mRNA encoding the Tol2 transposase [83]. Recently, a new technique had been established to make specifically targeted knockouts in endogenous zebrafish genes. By using engineered zinc finger nucleases (ZFN), targeted knockout mutations can be generated in zebrafish genes based on non-homologous end-joining-mediated repair of ZFN-induced DNA double-stranded breaks [84-86]. A final practical advantage of using zebrafish as a model organism is the fact that due to their small size they require relatively little housing space and maintenance costs are low.

1.3.2 Zebrafish as a new model to study vascular and lymphatic development

Zebrafish embryos, and more specifically the transgenic line *Fli1:eGFP^{y1}* which expresses GFP in all ECs [87], have been used extensively to study vertebrate vascular development. For a long time it was assumed that zebrafish did not have a lymphatic system. However, recently, several groups independently showed the presence of a

lymphatic system with physical and functional properties reminiscent of the mammalian system [6,7]. The thoracic duct (TD), which forms between the dorsal aorta (DA) and posterior cardinal vein (PCV), was the first identified lymphatic in zebrafish and has been reported to express Prox-1 and contain anchoring filaments [7,88]. This vessel also functionally drains interstitial dyes and has therefore been generally considered to be the first perfused lymph vessel in zebrafish [6,7]. Further studies in *Fli1:eGFP*^{y1} zebrafish embryos postulated that LECs arise primarily from venous BECs in the parachordal vessel (PAV) [7], which forms at the horizontal myoseptum from sprouts coming from the PCV [89]. However, a recent study has documented that the TD and other lymph vessels in the trunk arise from a non-lumenized aggregation of "parachordal lymphangioblast (PL)" cells (termed so because of their role as TD precursors); this structure, which transiently forms at the horizontal myoseptum, is distinct from the PAV, that develops only much later beyond 6 dpf [90]. High-resolution imaging indicates that this string of PL cells is anatomically transiently connected to the venous system. Indeed, of the secondary sprouts that arise from the PCV between 30 to 50 hpf, approximately half (on average one per two unilateral somite segments) migrate radially in the ventral-dorsal direction to the horizontal myoseptum (Figure 3A,A'); these sprouts exist only transiently [89,90]. At the horizontal myoseptum, the cells of these sprouts then migrate tangentially in the anterior-posterior direction to form the PL string (36 to 60 hpf; Figure 3B,B'). Since the secondary sprouts, which give rise to the PL string, participate in the process that ultimately leads to the formation of the TD, they have been termed "lymphangiogenic secondary sprouts" [90]. Indeed, the lymphangiogenic sprouts and PL cells give rise only to lymphatics but not to blood vessels, are not labeled in the arterial/venous *kdr-l:mCherryRed* marker line [90], and fail to form upon silencing of genes that regulate lymphangiogenesis in mice and humans, e.g., *Ccbe1* [90,91], *Vegf-c* [92], *Vegfr-3* [92] and *Synectin* ([93], manuscript under revision). However, we cannot clearly demonstrate that these structures already have a true lymphatic identity and express lymphatic marker genes (see Results). The other secondary sprouts from the PCV connect to the primary intersomitic vessels (ISVs) (Figure 3B), which thereby become intersomitic veins (vISV) (Figure 3C); these sprouts have therefore been termed "angiogenic secondary sprouts" [89,90].

From 60 hpf onwards, the PL cells switch to radial migration again, and navigate both ventrally and dorsally alongside arterial intersomitic vessels (aISVs), whereby they

form structures, that later persist as lymphatic intersomitic vessels (LISVs) (Figure 3C). These radially migrating cells will be termed LISV-PLs to distinguish them from the cells in the PL string. Once ventrally migrating LISV-PLs reach their final location in between the DA and PCV, they switch again to tangential migration, grow towards each other and fuse to establish the TD (3 to 6 dpf; Figure 3D,D'). While lymphangiogenic secondary sprouts migrate at a distance from and independently of aISVs [90], LISVs always navigate alongside aISVs, almost "creeping" over them in their initial dorsal and ventral trajectory, but never track alongside vISVs. This close association of LISVs with aISVs raises the question whether aISVs act as guidance templates for navigating LISVs-PLs, an issue that was also addressed in this thesiswork (see Results).

Until now, it has not been possible to unambiguously detect Prox-1 in the lymphangiogenic secondary sprouts or PL cells (likely because of insufficient sensitivity of the *in situ* technique in combination with very low expression levels), yet, a number of findings suggest a role of these structures in lymphatic development. Indeed, PL cells contribute only to lymphangiogenesis but not to angiogenesis, are not labeled in the *kdrl:mCherryRed* line which marks only arterial and venous endothelial cells (ECs) [90], and fail to form upon silencing of genes that regulate lymphangiogenesis in mice and humans, e.g., *Ccbe1* [90,91], *Vegf-c* [90] and *Prox-1* (unpublished). Since in the present project we were able to provide evidence for a role of Notch in lymphatic development, but not to provide unambiguous insight in the molecular basis of lymphatic competence, specification or differentiation, we refer to the lymphangiogenic secondary sprouts and PL cells as structures with a "lymphangiogenic" developmental potential (indicated by the light green color in Figure 3), without, however, claiming that they already are specified or differentiated to established lymphatic structures (indicated by the dark green color in Figure 3).

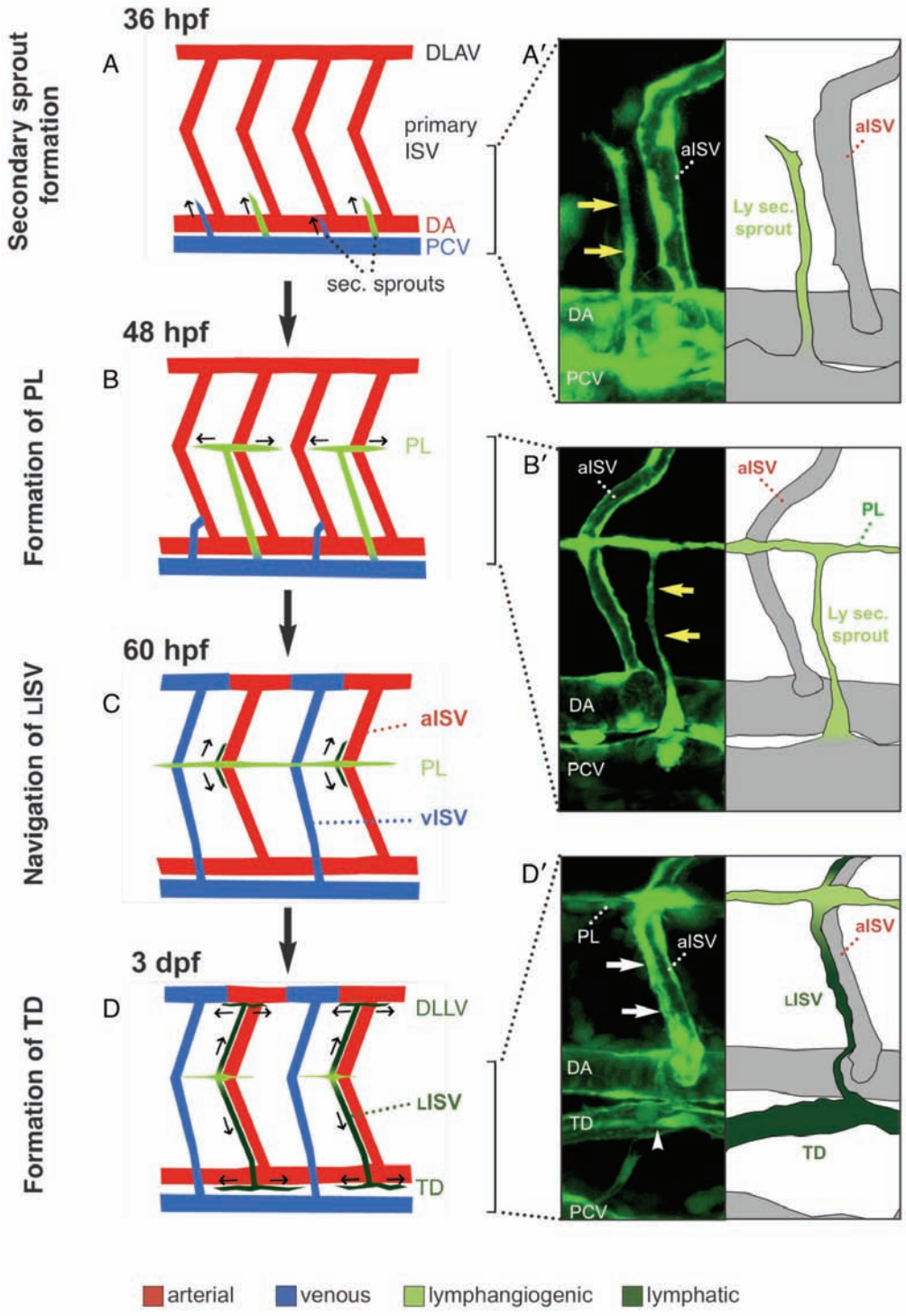

***Figure 3**: Schematic drawing of lymphatic development in zebrafish embryos. The schemes and photos show lateral views of a segment of the embryo trunk spanning 1-4 intersomitic vessels. For detailed description, see text. DA, dorsal aorta; DLAV, dorsal longitudinal anastomosing vessel; DLLV, dorsal longitudinal lymph vessel; ISV, intersomitic vessel; aISV, arterial ISV; LISV, lymphatic ISV; vISV, venous ISV; PCV, posterial cardinal vein; PL, parachordal lymphangioblast string; TD, thoracic duct. Permanent lymphatic structures (LISV, TD) are labeled dark green; transient lymphangiogenic structures (lymphangiogenic secondary sprouts; PL cells) are labeled light green.*

2 NOTCH SIGNALING

2.1 THE NOTCH SIGNALING PATHWAY

2.1.1 Pathway

The Notch signaling pathway defines an evolutionary cell interaction mechanism that was first described in Drosophila and is highly conserved from the nematode (roundworm) *Caenorhabditis elegans* to human. The *Notch* gene was discovered in 1917 by Tomas Hunt Morgan in a strain of the fruit fly *Drosophila melanogaster* with notches in their wingblades [94]. Sequencing of the gene and its molecular characterization was only undertaken in the 1980s [95,96]. The Notch gene encodes a 300kDa single-pass transmembrane receptor. It is composed of a hetero-dimer with a large extracellular portion that is non-covalently linked to a smaller piece that contains a short extracellular region, a single transmembrane-pass and a small intracellular region (Figure 4A). The two portions are translated as a single gene product but are posttranslationally cleaved by a furin-like convertase during intracellular trafficking in the Golgi complex [97,98]. The extracellular domain contains 29-36 tandem epidermal growth factor (EGF)-like repeats and three cystein-rich Notch/LIN-12 repeats. EGF11 and 12 are known to mediate ligand binding [99]. The intracellular domain contains six tandem ankyrin repeats, a glutamine-rich domain, a RAM23 domain (protein-protein interactions), a PEST sequence (proline-glutamate-serine-threonine-rich region) and a nuclear localization signal (NLS) [96]. In mammals four different receptors have been identified (Notch1, Notch2, Notch3, Notch4) whereas in zebrafish five homologues exist (Notch1a and Notch1b are homologous to Notch1, Notch6 is homologous to Notch2 and Notch5 is the homologue of Notch3, there is no homologue identified for Notch4) [100,101].

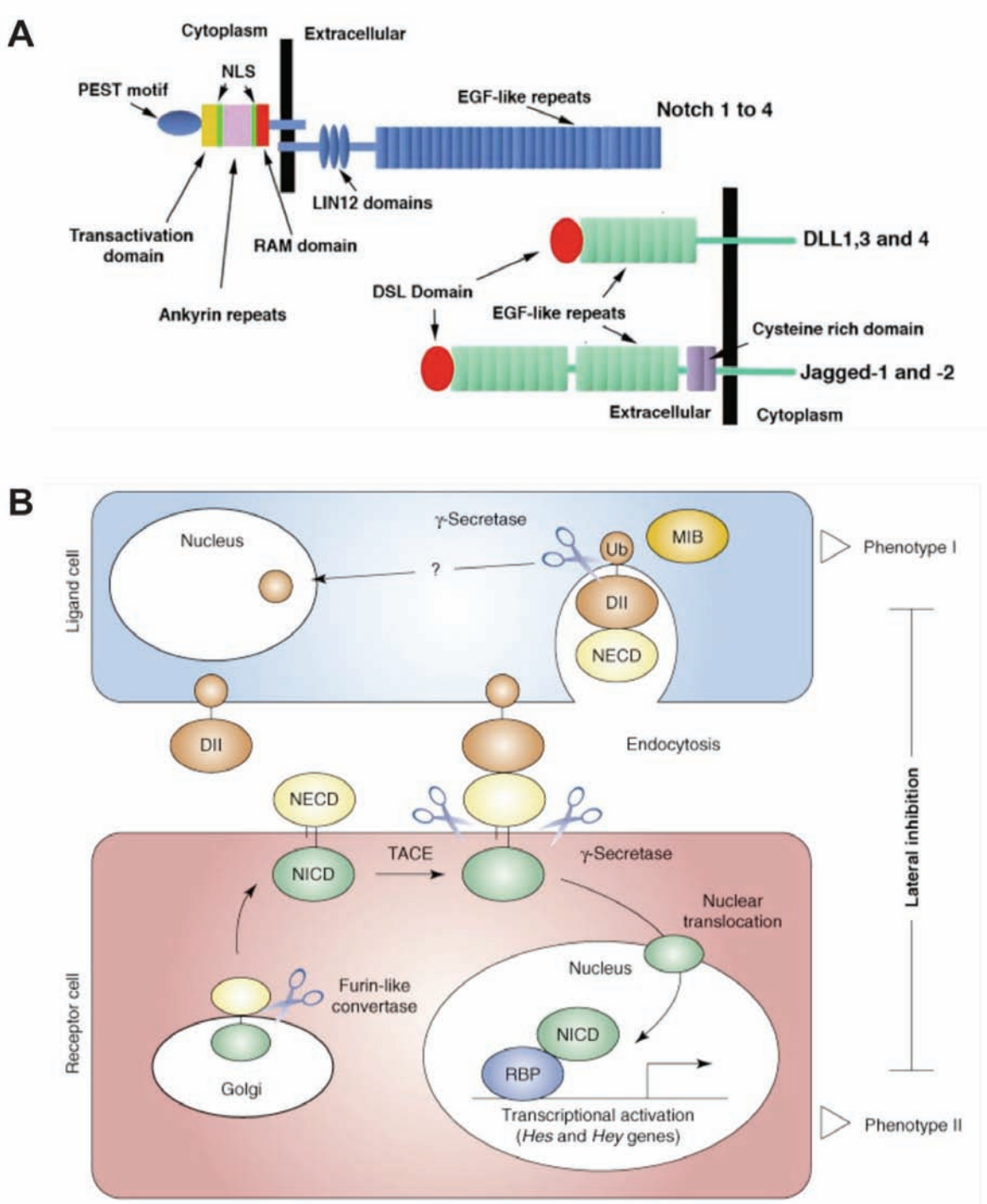

***Figure 4**: **The Notch signaling pathway (A)** General structure of the Notch receptors and ligands (from [102]). **(B)** Schematic overview of the canonical Notch signaling pathway (from [103]). For details: see text.*

The Notch receptors interact with transmembrane ligands of the Delta or Jagged family. Five ligands have been identified in mammalian cells (Delta-like1 (Dll1), Dll3, Dll4, Jagged1, Jagged2). In zebrafish eight ligands have been identified thus far (Delta-A, -B, -C, -D, Dll4, Jagged1a, Jagged1b, Jagged2). Interaction with the EGF-like repeats of the Notch receptor occurs through a conserved Delta:Serrate:Lag-2 (DSL) domain (Figure 4A). In theory all ligands can interact with all Notch receptors, however activity of the Notch receptor can by modified by *O*-fucosylation of the EGF repeats (mediated by *O*-fucosyltranserase-1) [104,105] and specificity of ligand-receptor interactions can be regulated by members of the *fringe* gene family [106,107]. Fringe acts in the Golgi apparatus as a β-1,3-*N*-acetylglucosaminyltransferase enzyme that further modifies the *O*-fucosylated EGF repeats of the Notch receptor and alters the ability of Notch to bind its ligand Delta. Generally, modification of the Notch receptor by Fringe makes it more sensitive to activation by Delta ligands and less sensitive to Jagged ligands [106]. There are three mammalian Fringe homologues: Manic Fringe, Lunatic Fringe and Radical Fringe [107].

2.1.1.1 Canonical Notch signaling pathway

Notch ligands and receptors are expressed in different combinations in most, if not all, cell types. Upon interaction of a receptor and a ligand on neighboring cells, proteolytic activation of the Notch receptor is triggered (Figure 4B). Endocytosis of the ligand on the signal-sending cell exerts a pulling force on the receptors in the signal-receiving cells, which enables processing of the Notch receptor by the activity of a disintegrin and metalloproteinase (ADAM) [108,109]. This initial extracellular proteolytic cleavage induces a second cleavage in the transmembrane domain by the γ-secretase complex, a large proteinase complex made of presenilin (PS)-1 or -2, nicastrin, Pen-2 and Aph-1). The result of both proteolytic cleavages is the release of the Notch intracellular domain (NICD) [110,111]. NICD is translocated to the nucleus where it interacts with the transcription factor of the CSL (CBF1, Suppressor of Hairless (Su(H)), Lag-1) family, also known as the recombination signal sequence-binding protein (RBP-jκ) [111] (Figure 4B). This interaction leads to removal of transcriptional repressors from and attraction of additional transcriptional activators to the complex, resulting in transcriptional activation of genes of

the Hes and Hey families [112] (Figure 4B). In turn, Hes and Hey proteins are transcriptional repressors that block the expression of genes that generally drive cells to adopt a certain differentiated fate.

It has been suggested that the endocytosis of the ligand may have consequences in terms of signaling in the ligand cell itself and that this leads to a reverse signaling mechanism [113-115] (Figure 4B). However, very little is known on the molecular mechanism and functions of this reverse signaling pathway.

2.1.1.2 Non-canonical Notch signaling pathways

In the vast majority of processes in which Notch signaling is involved, the Notch receptor is activated by a DSL-type ligand, which leads to changes in gene expression via binding of NICD to the CSL transcription factor. However, there are some exceptions where Notch signaling does not operate as this canonical trio.

Other proteins have been suggested to act as Notch ligands. F3/contactin and NB-3, two neural cell adhesion molecules, have been identified as alternative functional ligands of the Notch receptor [116,117]. Both ligands induce a downstream Notch signaling cascade via Deltex1 as a transcriptional regulator and mediate commitment of neural precursor cells to oligodendrocytes (OL), oligondendrocyte precursor cell (OPC) differentiation and OL maturation, whereas canonical DSL/Notch/CSL signaling regulates OPC generation and maintenance [116,117]. Besides a role of Deltex as transcription factor in a CSL-independent branch of Notch signaling [118], it can also promote Notch signaling by binding to the ankyrin repeats of the Notch intracellular domain and acting as an E3 ubiquitin ligase that promotes recycling of non-ligand bound endocytosed Notch receptors and antagonizes lysosomal degradation [119]. However, in some cells, including lymphoid cells and neurons, Deltex antagonizes Notch signaling, further complicating the picture and indicating that the function of Deltex may depend on the cellular and developmental context [120].

Several other examples of CSL-independent Notch signaling have been described. One of these is the interaction of activated NICD with the mTOR-Rictor complex (mammalian target of rapamycin (mTOR) and the rapamycin independent companion of

mTOR (Rictor)), which leads to the activation of the kinase Akt/PKB and inhibition of apoptosis in mammalian cells [121]. It has been shown that this function requires Notch processing as well as nuclear localization of NICD, but is independent of the CSL transcription factor [121]. The process of muscle cell differentiation is inhibited by Notch activation. However, this process does not require CSL-binding as mutant forms of Notch1 lacking the CSL interaction sequence were able to block myoblast differentiation and activated Notch1 did not induce CSL activation as shown by a CSL luciferase reporter assay [122]. Notch is present on growth cones of extending axons in *Drosophila* and can regulate axon guidance. It has been proposed that Notch may directly regulate the actin cytoskeleton via interactions with a protein complex that includes the axonal tyrosine kinase Abl [123]. Possibly Notch promotes axon extension by physically recruiting the Abl accessory protein Disabled, which binds directly to the intracellular domain of the Notch receptor [123]. The Abl tyrosine kinase and its associated proteins play a direct role in the regulation of growth cone motility during axonogenesis [124]. This requirement for Notch in axon patterning was shown to be genetically independent from its function in the control of cell identity [123].

2.1.1.3 Interaction of the Notch pathway with other signaling cascades

To add another level of complexity, the Notch signaling pathway interacts with many other signaling pathways like VEGF, Wnt, Hedgehog (Hh) and Transforming Growth Factor (TGF)-β / Bone Morphogenetic Protein (BMP) signaling. These complex and delicate interactions allow integration of these pathways into highly ordered and complex molecular networks that regulate embryonic development but also immune and vascular responses in the adult [125,126].

The cross-talk between the VEGF and Notch pathways is explained further in detail.

The Wnt signaling pathway is initiated by interaction of a Wnt ligand with its Frizzled receptor and leads to the stabilization of β-catenin by inhibition of glycogen-synthase kinase-3β (GSK3β). In the absence of activated Wnt signaling β-catenin is targeted for degradation by phosphorylation through a complex containing GSK3β, adenomatous polyposis colon protein (APC) and Axin-1. Stabilized β-catenin translocates to the nucleus where it forms a complex with transcription factors of the T cell factor (TCF) / Lymphoid

enhancer-binding factor (LEF) family and activates target gene expression (reviewed in [127]). An inhibitory cross-talk exists between the Notch and the Wnt signaling pathway at different levels. The Wnt ligand can bind to the extracellular domain of Notch [128], the Notch intracellular domain can interact with the intracellular Wnt pathway component Disheveled [129] and PS-1 interacts with β-catenin [130,131]. It has also been described that Wnt signaling induces Notch ligand expression and thus prevents Notch pathway activity in Wnt-activated cells [132].

In vertebrates three Hh ligands exist: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). In the absence of Hh ligands, the cell-surface transmembrane receptor Patched (Ptc) blocks the activity of Smoothened (Smo), another transmembrane protein. Binding of Hh to Ptc blocks its function, which allows Smo to accumulate and inhibit the proteolytic tranformation of Gli transcription factors from the activator form to the repressor form (reviewed in [133]). One of the downstream targets of Hh-activated signaling is the Wnt protein [134]. Both Notch and Hh signaling pathways are implicated in the regulation of differentiation and proliferation, but precisely how the two interact in regulating cellular processes is poorly understood. Depending on the cellular environment, their effects differ and their interaction can be positive or negative [135].

The TGF-β/BMP pathway involves TGF-β and BMP ligands, two types of receptors (type I and II) and the signal transducing Smads. On activation, the receptor complex phosphorylates and activates receptor-regulated Smads (R-Smads). The activated R-Smads interact with Smad4 and accumulate in the nucleus, where the Smad complex interacts with other factors to regulate target gene expression. TGF-β/BMP signaling is closely intertwined with the Wnt pathway, but also interacts with many other signaling pathways like Hh and Notch [136]. TGF-β can induce the expression of Notch ligands, and TGF-β/BMP and Notch synergistically regulate common target genes by direct interaction of NICD and Smad. These and other synergistic and antagonistic interactions of both pathways are reviewed in [136]

2.1.2 Functions

The Notch signaling pathway is important for cell-cell communication and is mainly involved in activation of gene regulation mechanisms to control multiple cell differentiation processes during embryonic and adult life. The best-known mechanism by which Notch regulates ***differentiation*** is via a process called 'lateral inhibition' (Figure 5A). This concept explains that when, within a pool of progenitor cells, a cell adopts a particular fate it inhibits its immediate neighbors from acquiring the same fate and instructs them to maintain their undifferentiated state. Initially, all progenitor cells are equivalent and express both Notch ligands and receptors. Due to stochastic reasons or intrinsic or extrinsic factors that are not always clear, one cell starts expressing higher levels of ligand than its neighbors. This cell becomes the 'signal-sending' cell that will adopt a certain phenotype and by activating the Notch signaling pathway in its neighbors prevents these 'signal-receiving' cells from acquiring the same phenotype. This is possible, due to the stabilization and amplifications of the differences in expression levels of ligand and receptor. Via a feedback-mechanism in the 'signal-receiving' cells, activation of the Notch receptor reduces ligand expression, thereby amplifying the initial small differences in levels of ligand expression and pushing these cells even more in a 'signal-receiving' mode and thus under control of the signals sent by the initial differentiating cell [137]. By activation of the Notch receptor, the 'signal-receiving' cells induce expression of Hes/Hey transcription factors, which are transcriptional repressors that prevent expression of differentiation-inducing factors. In this way only the few 'signal-sending' cells can adopt a differentiated state whereas the differentiation pathway is suppressed by Notch signaling in the surrounding cells. This process creates a 'salt-and-pepper' pattern of different cell types. Recent findings indicate that this model of lateral inhibition is too simplistic and that the pattern dynamically changes in time due to oscillations in Notch signaling [138]. An example of lateral inhibition is the maintenance of neuronal progenitors in the nervous system [137,138].

A second effect of Notch signaling is ***lineage decision*** making. A well-studied example is the B- *versus* T-cell fate decision in the immune system. This process is accomplished by asymmetric segregation of regulators upon division of a precursor cell into two daughter cells (Figure 5B). Examples of such regulators are Numb and Neuralized

(Neur). Numb inhibits the Notch receptor through a mechanism that involves endocytosis of the receptor [109]. Neur on the other hand is an E3-ligase that ubiquitylates the Delta ligand and promotes its endocytosis [139]. It has been suggested that this could enhance signaling by promoting the activation of Notch receptors. The asymmetric segregation of these regulators confers distinct cell fates to the daughter cells.

Lastly, Notch signaling can form ***boundaries*** between different regions or establish niches for maintaining specific cell types. An example is limb formation, where at the apex dorsal and ventral ectodermal cells confront each other and induce by Notch signaling formation of specialized cells that produce signals organizing outgrowth of the limb bud [140]. Another example is the formation of rhombomeres in the vertebrate hindbrain [141]. Somite formation seems to be a similar process in which boundaries are created. However, Notch plays multiple roles in this complex process that also differs between species. Underlying the process of segmentation there is a temporal oscillation in the expression of so called 'clock genes' in the unsegmented presomitic mesoderm at the tail end of the embryo [142]. The clock genes comprise components of the Notch, Wnt and FGF pathways. Especially Hes7 and Lunatic Fringe play important roles in regulating the oscillations through a negative feedback loop (Figure 5C), however, the nature of the ultimate generator and pacemaker of the oscillations is not entirely clear [143]. In mice, Notch signaling is required both for maintenance of the synchronic somitogenesis oscillation waves and for the formation of sharp somite boundaries [144]. In zebrafish embryos it was recently shown that Notch signaling functions only to synchronize the clock gene oscillations in neighbouring cells, but inhibition of Notch is not sufficient to interrupt the generation of a segmented body plan [145]. In mouse the sharp boundaries of Delta expression and Notch activation seem to be necessary to guide somite segmentation. In the anterior PSM, Notch activity and Lunatic Fringe waves are no longer regulated by Hes7, but become under the influence of the transcriptional regulator Mesp2 and a clear boundary is created where two non-equivalent groups of cells confront one another [146] (Figure 5C). Notch signaling from one group to the other group can confer a distinctive character on a band of cells along the boundary between the two populations. Finally, Notch signaling is also involved in the formation of the anterior-posterior polarity within each somite [147].

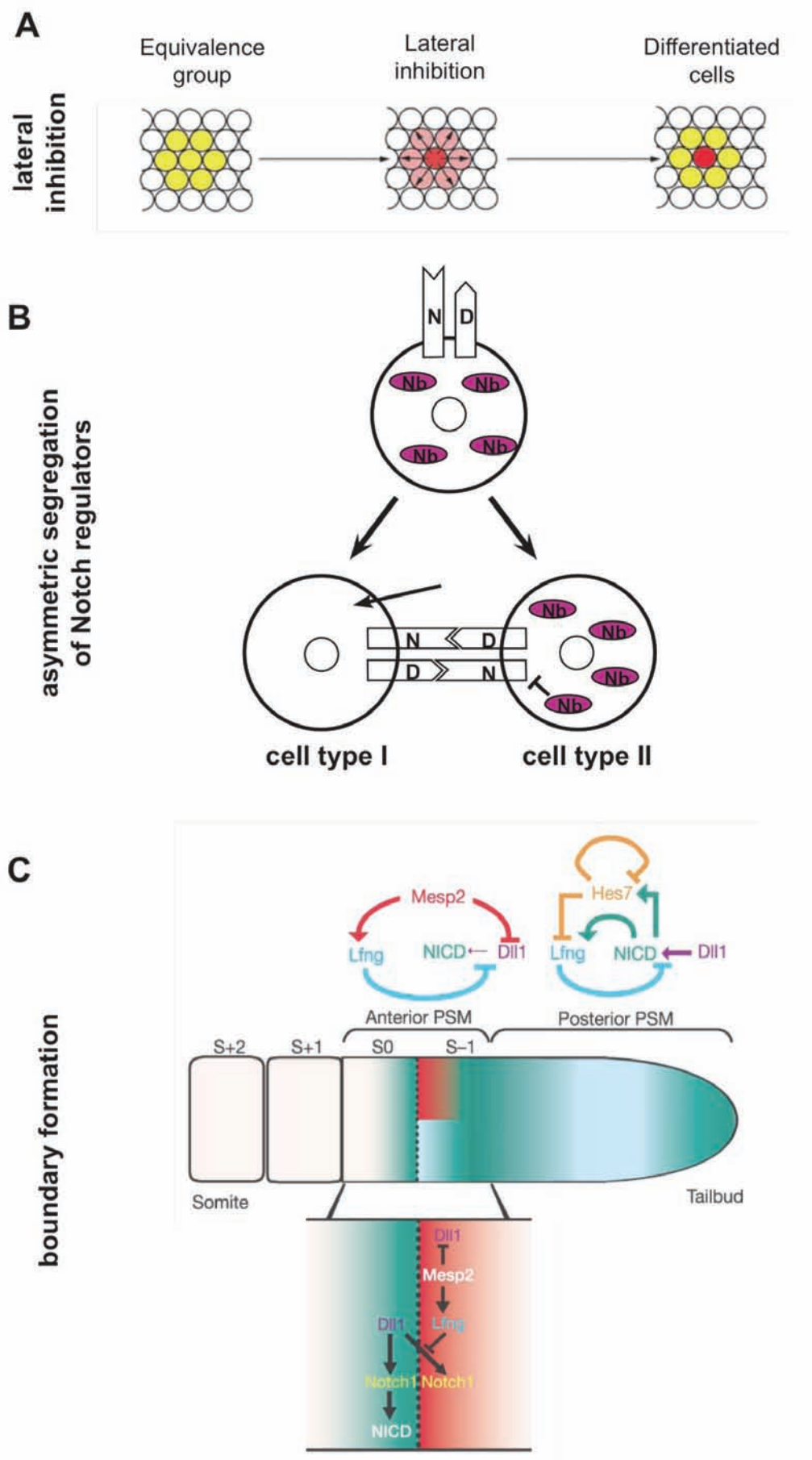
A
lateral inhibition
Equivalence group
Lateral inhibition
Differentiated cells
B
asymmetric segregation of Notch regulators
N
D
Nb
cell type I
cell type II
C
boundary formation
Mesp2
Lfng
NICD
Dll1
Hes7
Anterior PSM
Posterior PSM
S+2
S+1
S0
S–1
Somite
Tailbud
Notch1
NICD

***Figure 5**: **Notch signaling functions. (A) Lateral inhibition.** All cells within an equivalence group are in a particular state (yellow) and possess similar amounts of Notch and Delta. A combination of intrinsic and extrinsic signals induces a new cell state (light red) and breaks this balance by increasing the abundance of Delta in one or a few cells (dark red). Consequently, Notch signaling is activated in neighboring cells, suppressing the alternative fate and reverting these neighboring cells to their original fate through lateral inhibition. (adapted from scienceblogs.com/pharyngula/2006/12/notch.php). **(B) Lineage decision.** Regulation of Notch signaling by **asymmetric segregation** of Numb into the daughter cells upon cell division. Delta (D) / Notch (N) signaling occurs between the two daughter cells. Numb (Nb) inhibits Notch signaling and thus prevents Notch signaling in the Numb-containing daughter cell (adapted from [148]).**(C) Boundary formation.** In the posterior PSM, Notch signaling activates expression of Lfng and Hes7. Positive and negative feedback loops generate oscillations of Notch activity. In the anterior PSM, Notch activity and Lfng expression are regulatd by. As a result, Notch activity and Lfng waves are arrested and a boundary is created, which produces the next segmental boundary (adapted from [146]).*

2.2 NOTCH SIGNALING IN VASCULAR DEVELOPMENT

During the last decade, several studies have shown the importance of Notch signaling during different steps of vascular development [102]. The first evidence came from the fact that several components of the Notch pathway are expressed in the endothelium and in the surrounding mural cells. Notch1 is broadly expressed in many tissues, including the heart and vascular ECs [149]. Expression of Notch4 is mainly restricted to the vascular system [150-152]. Notch3 is predominantly expressed in vascular smooth muscle cells [153]. Four of the five mammalian Notch ligands (Jagged1, Jagged2, Dll1 and Dll4) are expressed in vascular ECs [149,154,155] and Jagged1 is also expressed in smooth muscle cells surrounding the arteries [156].

Further evidence for a role of Notch signaling in vascular development came from several *in vivo* studies in which genes of the Notch pathway were inactivated or overexpressed [102,157]. Homozygous deletion of *Notch1* in mice causes embryonic lethality as early as day E10.5 due to severe vascular remodeling defects in the yolk sac, the placenta and the embryo proper [149]. Targeted knockout in the endothelium causes the same phenotype [158]. Strangely, although Notch 4 is specifically expressed in the arterial endothelium [152], *Notch4*-null mice develop normally and are fertile. However, double

homozygous deletion of *Notch1* and *Notch4* causes vascular defects that are more severe than those observed in single *Notch1*-null mice [149]. Even more interesting is the fact that overexpression of an activated form of Notch4 in the endothelium leads to angiogenic defects similar to those observed in *Notch1/Notch4* double knockout mice [159] and in adult mice, expression of constitutively activated Notch4 is associated with arteriovenous malformations (i.e. abnormal connections between arteries and veins) [160]. Taken together, these findings indicate that the Notch signaling pathway needs to be finely tuned to regulate normal embryonic vascular development and that maintenance of proper Notch signaling is also required during adult life. Similarly, deletion of some of the Notch ligands is embryonically lethal. Mice lacking Dll1, Dll4 or Jagged1 die from vascular defects and severe haemorrhage [161-165]. Among these mutants, the Dll4 mutant displays the most severe vascular defects with deletion of even a single gene leading to lethality at E10.5 in most genetic backgrounds [161,162,164]. Interestingly, the only other gene that was previously described to show heterozygous lethality due to vascularization defects is VEGF-A [166,167]. Mutations in downstream Notch effectors also trigger vascular defects. Deletion of RBP-Jκ results in growth retardation by E8.5 and death by E10.5 due to abnormal placental development [168]. Mice lacking PS-1 and PS-2 or PS-1 alone exhibit vascular defects [169,170]. And finally, combined deletion of the Hey1 and Hey2 downstream Notch targets similarly results in vessel malformation [171].

Interestingly, all knockout mutants showed defective remodeling of the primary vascular plexus, indicating that Notch is dispensable for the initial vasculogenesis process (i.e. *de novo* formation of blood vessels from endothelial progenitor cells), but plays a crucial role during vascular remodeling through angiogenesis (i.e. sprouting from pre-existing vessels). These studies clearly demonstrate that Notch signaling plays a crucial role in angiogenesis. The molecular and cellular mechanisms by which Notch signaling regulates different steps of blood vessel formation are described in the following sections.

2.2.1 Arterial differentiation

Initial studies in zebrafish have shown that Notch signaling is essential for arterial differentiation [172-175]. The first indication came from a study of a mutant zebrafish strain

identified in an ENU-mutagenesis screening, the *gridlock (grl)* mutant, that lacks circulation in the trunk [175]. The *grl* mutation was identified and positioned to be in a gene homologous to the mouse *Hey2* gene. Gene expression analysis showed that *grl* is expressed as bilateral mesodermal stripes as early as the 10 somite stage, before vessel formation. Some of these *grl*-expressing cells migrate toward the midline to form the primordium of the DA at the 24 somite stage. By 30 somites, when blood begins to flow, *grl* is expressed strongly throughout the DA, whereas no *grl*-expressing cells are found in the vein [175]. Later on, the same group showed, by lineage tracking in zebrafish embryos, that angioblast precursors for the trunk artery and vein are spatially mixed in the lateral posterior mesoderm. Progeny of each angioblast however are restricted to either the artery or the vein, indicating that these early angioblasts are already fated to the arterial or venous identity. This arterial-venous decision is guided by a *notch-grl* pathway [174]. Knockdown of *grl* or ovexpression of a dominant-negative form of Su(H) (DNA-binding mutant Su(H)-DBM) specifically blocks formation of the DA and expanded the PCV [174]. Furthermore, these morphant embryos, as well as the *mindbomb (mib)* mutant fail to express the arterial marker genes *ephrinb2, notch5* and *deltaC* in the DA. In contrast, the venous markers *flt4* and *ephb4* are ectopically expressed in the DA. Remarkably, expression of other arterial marker genes, like *tbx20* and *grl* are not affected in these mutants. [172,174]. Activation of the Notch pathway by heat shock-inducible overexpression of the Notch1a intracellular domain in double transgenic *hsp70:Gal4;UAS:Notch1a-ICD* zebrafish embryos did not affect arterial marker gene expression but venous marker gene expression in the PCV was diminished [172]. Therefore, Notch signaling plays an important role in the expression of artery-specific genes and repression of a venous cell fate program within the DA. However, activation of the Notch pathway does not induce arterial cell fate identity ectopically, indicating that Notch signaling is cooperating with other pathways to mediate arterial differentiation. It was further shown that *Sonic hedgehog (Shh)* and *vegf* act upstream of the Notch pathway during arterial endothelial differentiation [173]. *Shh* is expressed in the notochord and induces expression of *vegf* in the somites at the time of angioblast migration. Vegf induces expression of *notch5*, thereby inducing arterial differentiation and blocking venous differentiation in a subset of angioblasts (Figure 6) [173,176]. A recent study shows that arterial and venous angioblasts initially form a single common precursor vessel and that the

embryonic artery and vein are formed by selective angioblast sprouting [177]. This sorting and segregation of arterial and venous angioblasts was shown to be regulated by a repulsive interaction of EphrinB2 and EphB4, which are expressed in arterial-fated and venous-fated progenitors, respectively, and orient the direction of progenitor cell migration [177].

Also in mammals Notch has been shown to be important for arterial development. Two Notch receptors, Notch1 and Notch4 are predominantly expressed in arterial ECs of early mouse embryos [149]. As described above knockout of *Notch1*, *Notch1;Notch4* or overexpression of an activated form of *Notch4* leads to embryonic lethality due to vascular defects [149,159]. Overexpression of constitutively active *Notch4* in adult mice leads to arteriovenous malformations that are accompanied by ectopic expression of the arterial marker *EphrinB2* in veins [160]. Dll4 is the only vascular specific ligand expressed in the DA at E8.5 and is thus believed to be the ligand for Notch1 and Notch4 during early vascular development. Dll1 is expressed in arterial ECs at a later stage (E13.5) and continues to be restricted to arterial ECs in adults and is required for maintenance of arterial identity [154,178].

Notch signaling is required for induction of arterial differentiation. However, venous differentiation is not a default pathway that is actively repressed by the Notch pathway to induce arteries. In order to obtain venous differentiation, the EC has to actively repress Notch signaling through COUP-TFII, a member of the orphan nuclear receptor superfamily, indicating that vein formation is also genetically programmed [179]. Endothelial-specific knockout of *COUP-TFII* leads to embryonic lethality by E12 due to vascular defects, including haemorrhage and thin and dilated vessels. Furthermore, mutant veins ectopically expressed *Jagged1, Notch1, Neuropilin-1 (Nrp-1)* and *EphrinB2* [179]. Endothelial-specific overexpression of *COUP-TFII* also induces angiogenesis defects and results in the formation of large, fused, disorganized vessels without arterial-venous distinction. It has therefore been proposed that COUP-TFII suppresses *Nrp-1* expression and inhibits Notch signaling in normal venous endothelium. Without the effects of Nrp-1 and Notch signaling, factors important for venous differentiation are expressed and venous identity is maintained. Removal of COUP-TFII activates the expression of arterial markers leading to acquisition of arterial identity [179].

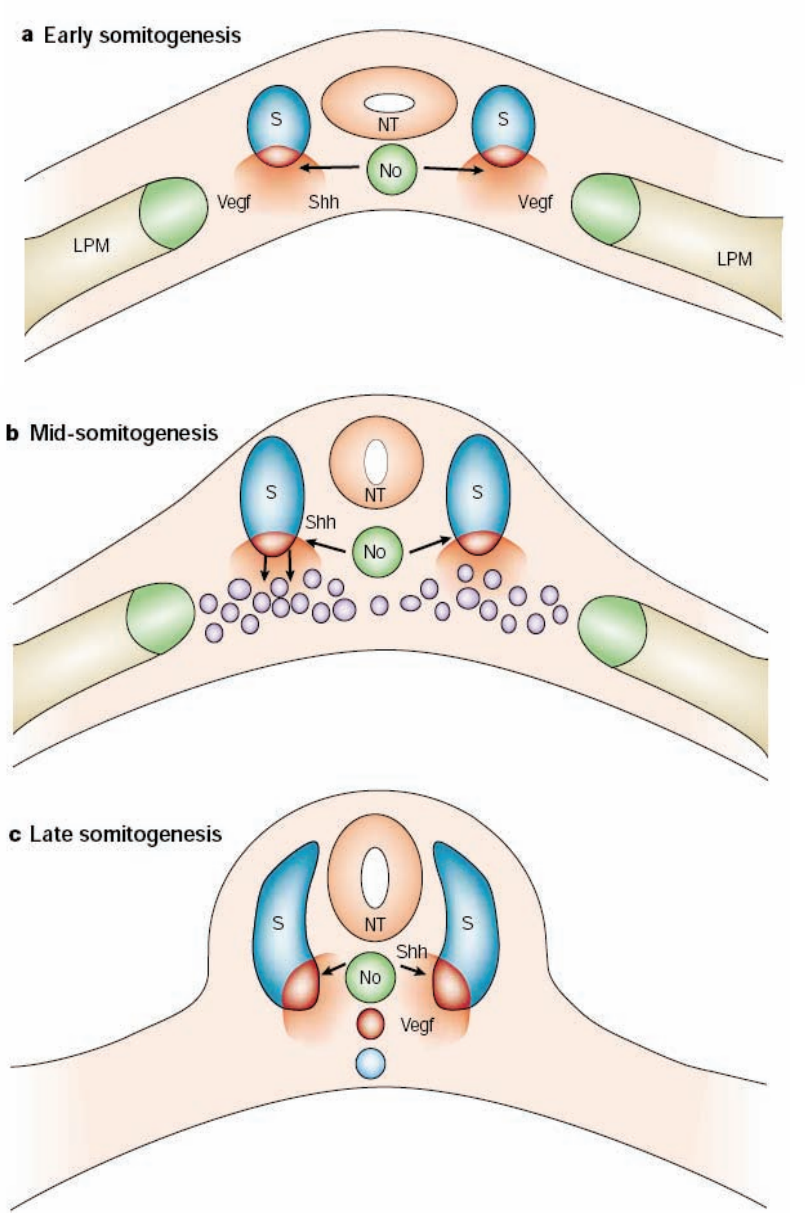

***Figure 6**: **A model for arterial differentiation in zebrafish** (from [176]). Schematic cross-sections through the trunk of a zebrafish embryo are shown. **(A)** At the 10-somite stage (ss), Shh expression in the notochord drives the expression of vegf in the surrounding somitic tissue. At this time, angioblasts arise from the dorsal lateral mesoderm (green) and come into close proximity to the source of Vegf. **(B)** By 18 ss, in mid-somitogenesis, local Vegf activates the Notch pathway, probably through the induction of notch5 in the developing dorsal aorta, therefore inducing the expression of ephrinb2a. Vascular progenitors migrate towards the midline, with the arterial progenitors leading the way (shown in purple). **(C)** By 24 h post-fertilization, in late somitogenesis, vein markers become downregulated in the arteries (red circle represents dorsal artery).*

LPM, lateral plate mesoderm; No, notochord; NT, neural tube; S, somite.

2.2.2 Tip cell selection

Angiogenesis is the sprouting of vessels from pre-existing ones. A newly forming sprout is composed of different endothelial cell types: tip cells and stalk cells (Figure 7) [180]. Tip cells continuously 'scan' the environment for guidance cues with their filopodia, long finger-like structures. In this way they can sense gradients of guidance cues and determine the direction of the growing sprout. Stalk cells are the cells trailing behind the tip cell and are responsible for elongation of the sprout. They proliferate, form junctions, lay down extracellular matrix and form a lumen. A third type of endothelial cells, the phalanx cells, are the quiescent cells lining the vessel once the new branches have consolidated [181]. They are termed phalanx cells because of their smooth cobblestone monolayer that resembles a phalanx formation of ancient Greek soldiers. They are covered by pericytes, stick to each other via tight junctions and are embedded in a thick basement membrane.

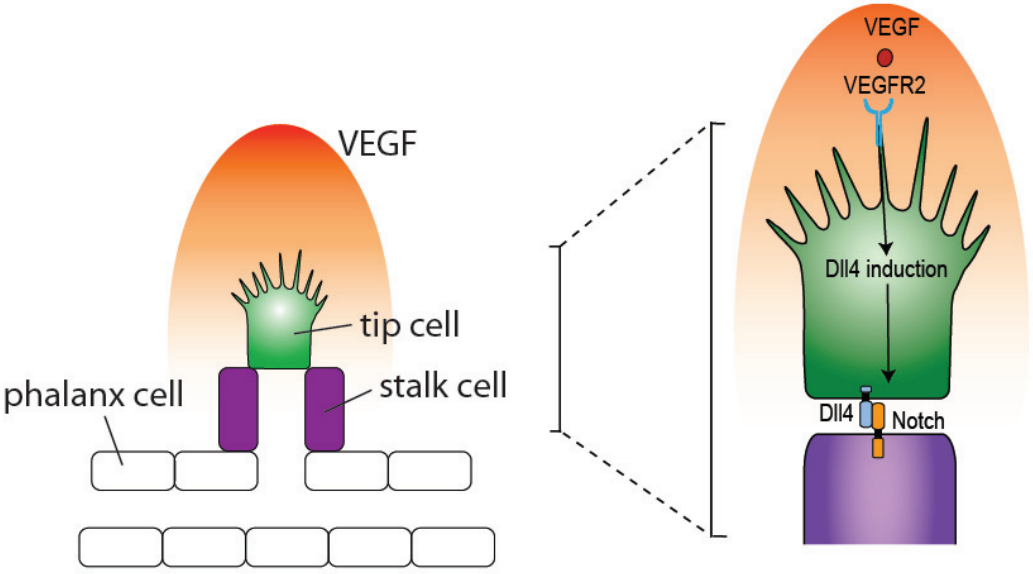

Figure 7: Schematic overview of a sprouting vessel. (left) *Different types of ECs in a vessel sprout growing towards a source of VEGF.* ***(right)*** *Molecular mechanisms regulating tip and stalk cell identity. (adapted from [180])*

Notch signaling is involved in the selection of ECs that become the leading tip cell during vessel branching. As described above, all ECs in a blood vessel express various Notch receptors and ligands. When a blood vessel is exposed to a VEGF gradient, the process of selection and induction of a tip cell begins [182]. The cell that becomes exposed to the highest level of VEGF will be selected to become a tip cell (Figure 7). By binding to VEGFR2, VEGF induces the expression of *Dll4*. Dll4 then binds to and activates the Notch receptor in the neighboring ECs, leading to downregulation of VEGFR2 in these cells. Thereby these cells become less responsive to the VEGF gradient and thus the VEGF-induced expression of Dll4 is dampened in these cells. This process is a clear example of lateral inhibition, whereby in a pool of equivalent ECs one cell is selected to become a tip cell that prevents its neighbors from becoming a tip cell as well and instructs them to be a stalk cell [183]. This molecular process was unraveled independently by several groups by studying the process of postnatal angiogenic sprouting in the mouse retina [184-187]. They all observed Dll4 expression at the leading front of the vascular plexus and found that inhibition of Dll4/Notch signaling resulted in an increased vascular density due to excessive sprouting. This hyperproliferation is dependent on VEGF and sprouting can be abolished by blocking VEGF/VEGFR2 interaction with antibodies or with soluble VEGFR1 [186,187]. Similar findings were described in the zebrafish ISV sprouting model where inhibition of the Notch pathway by DAPT treatment (a γ-secretase inhibitor that blocks Notch signaling by interfering with presenilin function [188]) or morpholino knockdown of Su(H) or Dll4 induces hyperbranching of the ISVs and leads to an increased number of ECs in the ISVs [189,190]. In contrast, inducible activation of the Notch pathway in the *Hsp70:Gal4;UAS:Notch1a-ICD* line blocks sprouting of the ISVs [190]. In zebrafish, Vegfr3 is expressed in the tip cells of the primary ISVs and knockdown of *Vegfr3* can partially rescue the Su(H) knockdown phenotype [190]. Later, also in mice, VEGFR3 was shown to be expressed in blood vessels undergoing angiogenesis, most strongly in the leading tip cells [191]. Dll4-induced Notch signaling downregulates *Vegfr3* expression and blocking VEGFR3 signaling can counteract hyperproliferation upon Notch inhibition [191]. Similarly in zebrafish, Dll4 functions to suppress the ability of developing intersomitic arteries to respond to Vegfc-driven Flt4 signaling and blocking Vegfc/Vegfr3 signaling rescues the Dll4 morphant hyperbranching phenotype in the ISVs [92]. More recently it was described that Jagged1 antagonizes the function of Dll4 in

sprouting angiogenesis [192]. This activity of Jagged1 depends on Fringe, which modifies the Notch receptor in stalk ECs and reduces activation by Jagged1, while enhancing Delta-mediated signaling. Jagged1 is expressed strongly in stalk cells but not or only weakly in tip cells. Jagged1 promotes angiogenesis by increasing tip cell numbers as an antagonist of Dll4/Notch signaling and prevents that low levels of coexpressed Dll4 in stalk cells can activate Notch in neighboring ECs [192]. In this way Dll4 and Jagged1 control the tightly balanced process of angiogenic sprouting.

Because of the crucial role of Dll4/Notch signaling in angiogenic sprouting, several groups have studied the role of this pathway in tumor development. The neovascularization of tumors through the angiogenic ingrowth of blood vessels from the surrounding tissue is one of the hallmarks of cancer that promotes tumor growth and metastasis. Dll4 is prominently expressed in tumor blood vessels in a VEGF-dependent manner [193-195]. Blocking Dll4/Notch signaling, either by a monoclonal anti-Dll4 antibody or by soluble Dll4 fused to the Fc domain of IgG, leads to increased vascular density within the tumor [186,194,196]. Paradoxically, despite this increased vascularization, tumor development is delayed [197]. This is because the resulting vessels are poorly functional, resulting in the tumors being more hypoxic [186,194,196,198]. These findings demonstrated that the stimulation of non-productive angiogenesis by blocking Dll4 could be used as a novel strategy for targeting tumor-induced angiogenesis, complementing anti-angiogenic therapeutic approaches [103]. However, despite the positive results of Dll4 blockade in preclinical studies, this novel anti-angiogenic strategy has very recently raised important safety concerns because of pathological activation of ECs, disruption of normal organ homeostatis and induction of vascular tumors [199]. Dll4 signaling appears to be essential for maintaining endothelium in the liver in a quiescent state and disruption of the pathway results in severe liver pathology [199]. Furthermore, chronic Dll4 blockade in rats results in subcutaneous tumors with features of vascular neoplasms, and lesions containing regions of necrosis can be found in heart and lung tissue [199]. Therefore, refined strategies might be necessary to exploit this pathway as a tool to disrupt tumor angiogenesis without causing prohibitive side effects.

2.2.3 Mural cell differentiation and maturation

Blood vessels not only consist of ECs, but also mural cells. The latter are derived from the mesenchyme and coat the endothelial cell tube. Mural cells can either be pericytes in the microvasculature or smooth muscle cells (SMCs) in the larger vessels. SMCs form multiple concentric layers around arteries and veins, whereas pericytes form a single, often discontinuous cell layer. Interactions between ECs and mural cells serve to stabilize nascent capillary vessels, provide survival factors for ECs, inhibit EC proliferation, promote mural cell differentiation and guide vascular network remodeling [200].

Several signaling pathways are involved in mural cell recruitment, maturation and differentiation: TGF-β, PDGF-B/PDGFR-β, EphrinB2, sphingosine-1-phosphate and Notch signaling [201,202]. The Notch3 receptor is highly expressed in pericytes and vascular SMCs of arteries and *Notch3*-null mice show defective maturation of SMCs and have enlarged vessels due to the lack of mural cells [202,203]. Similarly, patients with mutations in *Notch3* suffer from CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) syndrome, a pathology that causes stroke and dementia and is associated with degeneration of SMCs [204]. Also modulation of Dll4 signaling in tumor models has been described to result in deficient pericyte coverage [196,198]. Possibly this is caused by decreased EphrinB2 expression in pericytes which is required for their recruitment to vessels [205-207].

Endothelial cell - mural cell contact induces expression of Notch3 in mural cells, which is dependent on expression of Jagged1 in ECs [156]. Indeed, endothelial-specific deletion of Jagged1 results in striking deficits in vascular SMCs confirming the important role of endothelial Jagged1 to regulate the development of neighboring SMCs [208]. The increased expression of Notch3 in mural cells is accompanied by upregulation of SMC differentiation marker genes. However, this effect is only observed in mural cells that already express SMC marker genes, indicating that activated Notch signaling upon contact with an EC partner induces proper SMC maturation [156]. *Notch3*-negative arterial SMCs are altered in size and shape and fail to properly associate with ECs. Proliferation and survival however are not affected [203]. PDGFRβ was shown to be a direct Notch target gene [209]. PDGF-B/PDGFR-β signaling is required for recruitment of mural cells. PDGF-B is secreted by the

endothelium of angiogenic sprouts and is bound to the extracellular matrix where it serves as an attractant for migrating pericytes, which express PDGFR-β. In addition, PDGF-B stimulates proliferation of SMCs and induces mural cell fate in undifferentiated mesenchymal progenitor cells [210]. Therefore it is suggested that Notch affects maturation of mural cells indirectly via its effects on PDGF signaling [209].

Very recently it was described that Notch signaling together with TGF-β signaling is also required for differentiation of mesenchymal stem cells into SMCs [211]. Both TGF-β and Notch activation induce the upregulation of SMC marker gene expression. More specifically, TGF-β induces the expression of Jagged1 in mesenchymal stem cells, which is necessary for the upregulation of various SMC markers. This suggests that Notch signaling mediates TGF-β regulation of mesenchymal stem cell differentiation into SMCs [211].

2.2.4 Role of Notch in postnatal vascular development

From the above it is clear that the Notch signaling pathway plays a crucial role during different steps of embryonic vascular development, but Notch signaling was also found to be essential for vascular development during postnatal and adult life. Blood vessel growth is induced postnatally under different physiological and pathological conditions, like wound healing, inflammation, ischemia, cancer, etc. Blood vessels in adult tissues are mainly formed through angiogenesis, the sprouting of pre-existing blood vessels. However, there is scientific debate about the contribution in adults of vasculogenesis, the process by which progenitor stem cells differentiate to ECs and give rise to *de novo* formed blood vessels and that occurrs during embryonic life [212]. Bone marrow-derived cells have been identified in peripheral blood in adults and shown to participate in new vessel formation [213,214]. These cells were termed endothelial progenitor cells (EPCs). Despite the controversy concerning the existence of EPCs (see further), a role for Notch has been described in both postnatal neoangiogenesis and angiogenesis through sprouting.

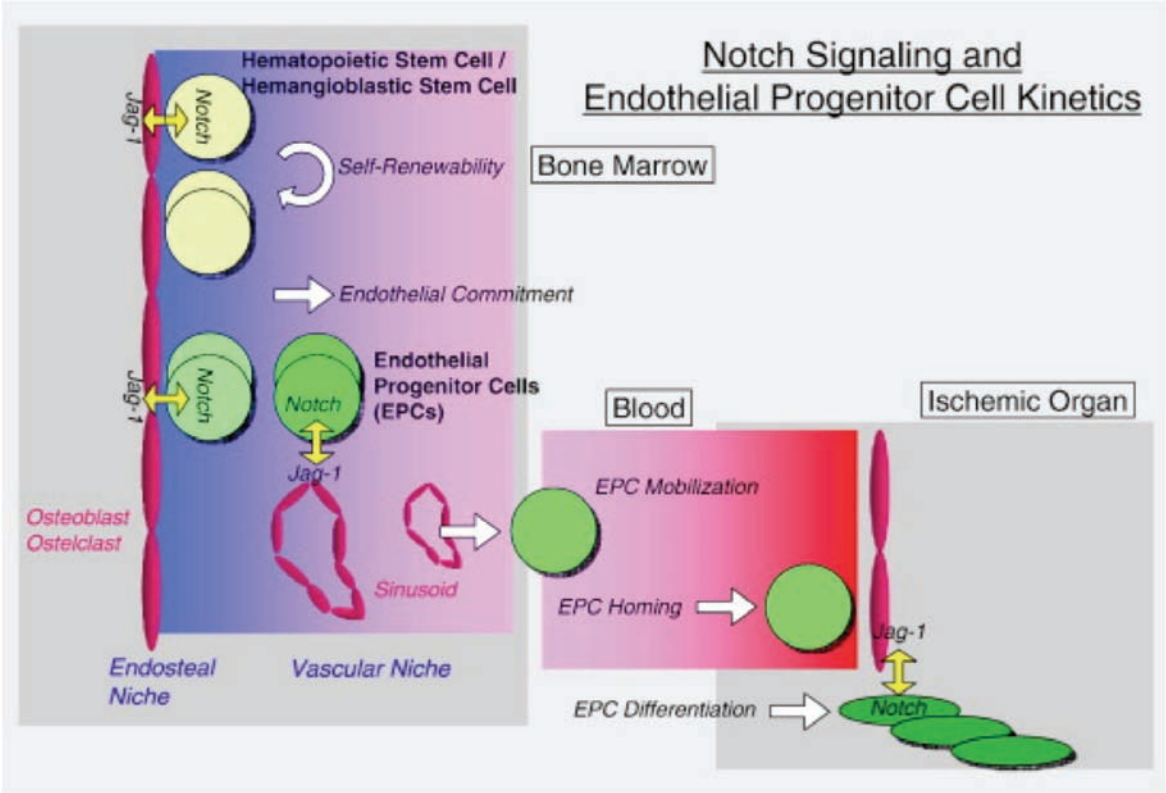

***Figure 8**: Notch signaling in EPC-mediated neovascularization. In the BM niche, Jagged-1 is expressed in stromal cells and modulates the development and functional kinetics of EPCs, which express Notch receptors. This interaction influences EPC proliferation, differentiation, mobilization, and ultimately EPC-mediated active neovascularization in ischemic sites. (from [215])*

In the bone marrow (BM), EPCs differentiate from hemangioblastic stem cells, a common stem cell for EPCs and hematopoietic stem cells (HSCs) [216]. They are mobilized into the blood stream upon signaling by cytokines and growth factors, such as granulocyt-macrophage colony stimulating factor (GM-CSF) and VEGF-A, and are recruited to sites of hypoxia or wounding where they interact with tissue-specific cells and contribute to the formation of new blood vessels. In the BM niche, Notch ligands, especially Jagged-1 and Dll1, are expressed in osteoblasts, stromal cells and ECs [217,218] (Figure 8). The interaction between osteoblasts expressing Notch ligands and HSCs expressing Notch receptors is one of the molecular mechanisms regulating HSC self-renewal and maintenance [219]. Similarly, Notch signaling is involved in EPC biology in the BM niche [220]. It was shown that

loss of Jagged-1 ligand in mice, but not Dll1, leads to an impairment of proliferation, survival, differentiation and mobilization of BM-derived EPCs, resulting in a reduced therapeutic capacity of EPCs for vasculogenesis and vascular regeneration in ischemic tissues [220]. Therefore, signaling between stromal cells expressing the Notch ligand Jagged-1 and EPCs expressing Notch receptors seems to be essential to drive immature BM cells to commit and differentiate into functional EPCs. Furthermore, Notch signaling may not only be involved in the interaction of EPCs with the BM niche, but also play pivotal roles in the peripheral tissues by regulating homing, on site differentiation and integration of EPCs into the target tissues [215].

Recent discoveries in both mice [221] and humans [222] have led to questions and discussions about the existence of EPCs. In contrast, other recent evidence indicates that EPCs are important in tumour angiogenesis and metastasis [223,224]. Further, despite the findings of some studies that have shown a correlation between low numbers and funcitionality of EPCs and higher risk of cardiovascular disease, therapeutic strategies using transplantation of BM-derived cells has shown at best only moderate effects (reviewed in [212]). It has been suggested that the described EPCs may indeed contribute to the angiogenic processes but are not real progenitors of adult endothelium [212]. Rather, the primary contribution of these BM-derived monocytic cells is not by incorporating themselves into the newly forming vessels, but by migrating to the site of neoangiogenesis where they deliver signals that activate pericytes, which in turn secrete mediators that stiumulate migration and proliferation of local ECs [225]. Nevertheless, Notch signaling plays a critical role in mobilization of BM-derived cells, whatever role these cells play at the site of neovascularization.

Besides its function in neovascularization, Notch signaling also plays a role in both physiological and pathological postnatal angiogenesis whereby sprouting occurs from existing blood vessels, for example during hypoxia, wound healing, inflammation and tumor growth. In a model of hindlimb ischemia it was shown that blood flow recovery and neovascularization were impaired in global or endothelial specific heterozygous Notch1$^{+/-}$ mice, but not in Notch4$^{-/-}$ mice [226]. Upon ischemic injury, Notch1 expression is induced in the endothelium. VEGF is well known to be upregulated in response to hypoxia and to promote

angiogenesis in ischemic tissues [227]. Through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, VEGF induces γ-secretase mediated cleavage and activation of Notch1, which is critical for VEGF-induced EC proliferation, migration and survival [226]. The Notch ligand Dll1 has also been described as a critical regulator of postnatal angiogenesis [178]. Its expression in the arterial endothelium is strongly upregulated during ischemia and heterozygous $Dll1^{+/-}$ mice, which survive to adulthood, show reduced formation of collateral arteries and fail to restore blood flow in a hindlimb ischemia model, similar to the $Notch1^{+/-}$ mice [178].

Similar to developmental angiogenesis, Notch signaling is involved in tip cell selection during postnatal angiogenesis to control sprouting of new vessels from pre-existing ones. As described above, during development, this process is critically regulated by Dll4-mediated Notch signaling. During inflammation-induced angiogenesis however, an upregulation of Jagged1 was observed in ECs becoming tip cells [228]. Jagged1 expression is induced by the inflammatory cytokine tumor necrosis factor (TNF) through an NFκB-dependent mechanism. Paradoxically, at the same time TNF blocks VEGFR2 signaling, thus blocking the VEGF-driven angiogenic response. However, this concomitant tip cell selection and angiogenesis inhibition functions to prime ECs for sprouting once the initial inflammatory wave has passed [228]. These findings suggest that during inflammatory angiogenesis Jagged1 plays a role similar to that of Dll4 during developmental tip cell selection. Tumor angiogenesis may represent a mixture of physiologic and inflammatory angiogenesis as both Dll4 and Jagged1 are upregulated on tumor vasculature [195,228]. The effects of blocking Dll4/Notch signaling during tumor-induced angiogenesis are described above.

2.3 NOTCH SIGNALING IN LYMPHATIC DEVELOPMENT

Intriguingly, despite the venous origin of lymph vessels, several molecular players, involved in arterial EC fate specification, have been implicated in lymphangiogenesis. EphrinB2 is initially a specific marker of arterial ECs [172], yet, becomes a lymphatic marker later in development, and regulates lymphangiogenesis [70]. The forkhead transcription factors, Foxc1 and Foxc2 are required for arterial cell fate specification, but subsequently regulate sprouting of LECs from veins [69]. Foxc2 is also involved in maturation of lymphatic

vessels. It is expressed in the luminal valves and loss of function results in failure to form valves and recruit SMCs to lymphatic collecting ducts [68]. Remarkably, both EphrinB2 and the Foxc1/2 transcription factors can be linked to the Dll4/Notch signaling pathway. Notch regulates arterial differentiation, in part through upregulation of EphrinB2 expression in arterial ECs [172]. Moreover, *in vitro* co-culture experiments have shown that specifically Dll4 stimulation, but not Jagged1 markedly induced EphrinB2 expression in venous ECs [207]. Foxc1 and Foxc2 were shown to induce the expression of Notch1, Notch4, Dll4 and Hey2 [69,229]. The regulation of Dll4 and Hey2 occurs via direct interaction of the Foxc transcription factors to Foxc-binding sites in both promoters [69,229]. The activity of Foxc proteins in the activation of the Dll4 and Hey2 promoters is further enhanced by VEGF-mediated PI3K and ERK/MAPK pathways [229]. Furthermore, $Foxc1^{-/-}$;$Foxc2^{-/-}$ double knockout mice exhibit arteriovenous malformations and loss of arterial marker gene expression [69]. Taken together, it seems that the Foxc transcription factors act upstream of Notch and EphrinB2 signaling in the regulation of arterial specification. The involvement of both Foxc1/2 and EphrinB2 in lymphatic development and their regulatory relationships to Notch signaling in arterial development raises the question whether Notch signaling is also involved in lymphatic development and even more, whether Notch signaling is generally involved in differentiation away from the venous identity towards either an arterial or lymphatic fate, possibly dependent on co-regulation by other thus far unknown factors. Very recently, Notch1 and Notch4 were found to be expressed in normal and tumor LECs and Notch1 is activated in lymphatic endothelium of invasive mammary micropapillary carcinomas [230]. Furthermore, cultured LECs express some of the Notch receptors (Notch-1, -2 and -4), ligands (Dll1, Jagged1, Jagged2) and downstream targets (Hey1 or -2) [231,232].

As described above Notch signaling induces arterial differentiation and suppresses venous identity [172]. COUP-TFII directs differentiation towards a venous fate by blocking Notch signaling [179]. At a later stage venous ECs can differentiate into lymphatic ECs by expression of additional key regulators like Sox18 and Prox1 [39]. Interestingly, the venous regulator COUP-TFII also appears to be essential for LEC specification and maintenance of the LEC phenotype [233,234]. Its expression was shown in cardinal vein-derived sprouting LECs and newly formed lymphatic vessels in mouse embryos [233,234]. COUP-TFII physically interacts with Prox1 to form a transcription factor complex that regulates several lineage-

specific genes including VEGFR3, FGFR3 and Nrp-1 [233,234]. In most cases both transcription factors were found to co-regulate expression of their target genes, however, they can also counter-regulate target gene expression, as was shown for Nrp-1 [233]. Furthermore, the effect of COUP-TFII on Prox1-mediated regulation of VEGFR3 was shown to be different in BECs (negative) and LECs (positive), indicating the high level of complexity and cell type-dependency of this system [234]. The co-regulatory effect of Prox1 and COUP-TFII on the expression levels of Notch pathway components has not been described.

Vascular-specific deletion of COUP-TFII blocks venous differentiation and LEC formation [35,235]. Recently, it was shown that the lost venous identity, but not the loss of LECs, can be rescued by simultaneous deletion of Rpbj [235]. These findings seem to indicate that, unlike arterial-*versus*-venous specification, lymphatic-*versus*-venous specification is not dependent on canonical CSL-dependent Notch signaling [235]. However, it should be noted that only a few of the Rpbj-*null* mice generated in this study survived, possibly pointing to an incomplete deletion of Rbpj in the analyzed mouse embryos, which might be sufficient for LEC specification. The same group showed that COUP-TFII is involved in a three-phased regulation of Prox1 expression [235]. The initiation of Prox1 expression was shown to be dependent on COUP-TFII expression. The subsequent maintenance phase of Prox1 expression is dependent on the interaction of Prox1 with another nuclear receptor. Most likely a Prox1-COUP-TFII interaction is required since COUP-TFII was shown to be required also for maintenance of Prox1-expression in early differentiating LECs. Finally, later maintenance of Prox1, beyond E13.5, becomes independent of COUP-TFII [235]. However, another study showed that conditional loss of COUP-TFII at E12.5 leads to loss of lymphatic markers and upregulation of BEC markers, but expression of Prox1 was unchanged [236]. Thus, this indicates that already at E12.5 maintenance of Prox1 expression is COUP-TFII independent and that COUP-TFII regulates LEC identity independent of Prox1 [236]. Further, COUP-TFII is also involved in VEGF-C-induced lymphangiogenic activities, including proliferation and migration and this role is mediated, at least in part, by direct stimulation of Nrp-2 expression [236]. Finally, COUP-TFII also seems to be essential for pathological neo-lymphangiogenesis in adult mice as inactivation of COUP-TFII in a tumor model results in inhibition of tumor lymphangiogenesis [236]. Another group [237] recently investigated the regulatory relationship among the three endothelial cell fate regulators

(Notch, COUP-TFII and Prox1), which remarkably co-reside in LECs [45,230,233]. They found that these regulators are under an exquisite feedback control mechanism and dynamically cross-regulate each other in established LECs [237]. This finding could explain the remarkable cell fate plasticity of LECs [46-48,238,239]. Notch signaling represses the expression of Prox1 and COUP-TFII in post-developmental LECs. In turn, Prox1 and COUP-TFII repress VEGFR-2 and Nrp-1, thereby attenuating VEGF signaling, which is known to induce the Notch pathway [237]. The authors propose that LEC fate may not be governed by a two-way turn ON-OFF switch, but rather by a dial switch that allows a gradient increase or decrease in the LEC fate force [237]. The implications for and interpretation of these recent findings in light of our own data have been thoroughly discussed in the Discussion section.

Another interesting link between Notch signaling and lymphatic structures was found in Kaposi's sarcoma pathology. Kaposi's sarcoma is a type of cancer affecting lymphatic endothelium that is caused by infection with the Kaposi sarcoma herpes virus (KSHV). Infection of BECs with KSHV leads to their lymphatic reprogramming with induction of lymphatic-specific genes and downregulation of blood vascular genes [238,239]. Recently it has been shown that KSHV-infection of LECs induces expression of Dlll3, Dll4, Jagged1, Notch3 and Notch4 and the Notch target genes Hes1 and Hey1 [240,241]. A role for Dll4 and Jagged1 was suggested in the pathogenesis process by suppressing the cell cycle in adjacent non-infected LECs, thereby providing a growth advantage to infected over uninfected LECs [240]. However, another study describes induction of expression of the endothelial precursor cell marker CD133 and several mural cell markers after KSHV infection of LECs, suggesting dedifferentiation and trans-differentiation processes [241]. In any case, these studies indicate a potential role for Notch signaling in LECs in regulating proliferation and/or differentiation processes.

Despite all these indications of a possible role for Notch signaling in lymphangiogenesis, so far, no lymphatic phenotype was reported in mouse embryos carrying homo/heterozygous deletions of different Notch signaling components that are involved in vascular development (Notch1 [164], Notch1/Notch4 [164], Jagged1 [165], Dll4+/- [161,162,164], Hey1/Hey2 [171], PS1/PS2 [169,170]). However, these mutant mice die due to vascular defects at E9.5-10.5, a time point at which lymphatic development is only just initiated. In

this study we have therefore analyzed the effects of inhibiting Notch signaling on lymphatic development in the zebrafish and tadpole models. These small aquatic animals are less dependent on normal vascular development and can survive several days without a fully functional circulatory system, thus allowing the analysis of the lymphatic developmental process after Notch inhibition.

Chapter II

AIMS

The relationship between "arterial" factors and lymphangiogenesis, as well as the anatomical congruence between arteries and lymphatics (see Introduction p 40) prompted us to investigate whether Notch signaling, known to induce arterial endothelial fate [172-174,177,240,242-244], also regulates lymphatic development. Notch and its ligand Dll4 seemed intriguing candidates, given their well-described role in vessel branching [184,187,189,190,194,245].

The general aim of this thesis was therefore to determine whether the Notch signaling pathway in general, and more specifically Dll4/Notch signaling, is involved in lymphatic development, and if so, to assess which steps during the lymphangiogenesis process are regulated by this pathway.

AIM 1: Does inhibition of Notch signaling interfere with normal lymphatic development in the zebrafish and Xenopus model?

To answer this question we first performed general inhibition of the Notch pathway by means of the chemical compound *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) in zebrafish embryos and tadpoles. DAPT is a well known γ-secretase inhibitor, that blocks all canonical Notch signaling. The compound was added to the tank water of the embryos at early developmental stages, and the effect on lymphatic development was followed over time. In the tadpole model, the formation and functionality of the caudal lymph vessels was studied. In the zebrafish model, the formation of the thoracic duct was analyzed in these initial experiments, since this is the major lymphatic vessel in the zebrafish trunk, that is easily observable, and since this is the ultimate outcome within the first 5 to 6 days of normal lymphangiogenesis.

Secondly, we more specifically studied the involvement of the different Notch pathway components by performing a morpholino knockdown screening in zebrafish, targeting the different Notch receptors (Notch-1a, -1b, -5, -6) and ligands (Delta-A, -B, -C, -D, Dll4) known in zebrafish and several of the downstream effectors (PS-1, PS-2, Su(H)a/Su(H)b).

AIM 2: What is the mechanism by which Notch signaling is affecting lymphangiogenesis?

After having observed an effect of Notch inhibition on lymphatic development, the next step was to determine exactly which steps of the lymphatic developmental process are regulated by Notch signaling. For those morpholinos that impaired thoracic duct formation, the earlier steps of lymphatic development were studied. Earlier steps entail (i) parachordal lymphangioblasts (PL) string formation, (ii) lymphangiogenic sprouting from the PCV to form this PL string and concomitant formation of arterial or venous ISV connections, and (iii) navigation of LISV-PLs from the PL string to the TD position along their arterial template (Figure 3).

Further *in vitro* experiments were performed to determine if blocking Notch signaling affects venous-to-lymphatic differentiation, LEC migration, proliferation or sprouting.

Chapter III

MATERIALS & METHODS

1 ZEBRAFISH EXPERIMENTS

1.1 HUSBANDRY & TRANSGENIC LINES

Adult fish were maintained under standard laboratory conditions. Embryos were kept at 28°C in 0.3x Danieau (embryo water) supplemented with phenylthiourea (PTU) to prevent pigmentation [246].

Transgenic zebrafish lines used were *Fli1:eGFP*^{y1} [87], *Flt1:YFP* [90], *kdr-l:mCherryRed* [90], *Tp1:eGFPxFli1:DsRed* [247] and intercrosses.

1.2 COMPOUND INHIBITOR TREATMENT

A 10 mM stock solution of *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; γ-secretase inhibitor IX; Calbiochem) in DMSO was diluted in embryo water to the indicated treatment concentrations. Embryos were dechorionated by trypsinization (Sigma, 1.5 mg/ml in PBS) at 24 hpf and incubated in 6.25 to 25 μM DAPT in embryo water at different stages between 24 and 72 hpf. DMSO was added to the lower concentrations to equalize total DMSO to 0.25% in all conditions. Control embryos were incubated in 0.25% DMSO in embryo water.

1.3 MORPHOLINO INJECTION OF ZEBRAFISH EMBRYOS

Gene-specific antisense morpolino oligos were purchased from Gene Tools (LLC, Corvallis). Sequences are listed in Table 1. For reasons of consistency, we used the zebrafish nomenclature [173]: zebrafish Notch-5 and -6 are mammalian homologues of Notch-3 and -2, respectively, while zebrafish Notch-1a and -1b are duplicated mammalian orthologues of Notch-1 [100,101]. For the previously unpublished morpholinos, silencing efficiencies of morpholinos directed against the ATG region were determined as previously described using an *in vitro* luciferase reporter assay [32] and were confirmed (not shown). Morpholinos were injected into single- to four-cell stage zebrafish embryos, using procedures as previously described [90,246] and embryos were further incubated in embryo water at 28°C until 4 - 12 dpf with feeding from 7 dpf onwards. All data shown in the

manuscript were obtained after injection of 1.5 to 20 ng of morpholino, as indicated. Control embryos were injected with control morpholino (Gene tools, LLC) (Table 1)or injection buffer (0.1% phenol red).

TABLE 1: MORPHOLINO OLIGONUCLEOTIDE SEQUENCES

MO	MO sequence	Target	Ref
DeltaA^{ATG}	5'-CGCCGACTGATTCATTGGTGGAGAC-3'	Start site	
DeltaB^{ATG}	5'- CGCCATCTCGCTCACTTTATCCTAA-3'	Start site	
DeltaC^{ATG}	5'- GCACGTTAATAAAACACGAGCCATC-3'	Start site	
DeltaD^{ATG}	5'- AACAGCTATCATTAGTCGTCCCATG-3'	Start site	
Dll4^{ATG}	5'-GAGAAAGGTGAGCCAAGCTGCG-3'	Start site	
Dll4^{SPL}	5'-TAGGGTTTAGTCTTACCTTGGTCAC-3'	Exon6/intron6	189
Jag1a^{ATG}	5'-GTCTGTCTGTGTGTCTGTCGCTGTG-3'	5' UTR	
Jag1b^{ATG}	5'-CTGAACTCCGTCGCAGAATCATGCC-3'	Start site	
Jag2^{ATG}	5'-TCCTGATACAATTCCACATGCCGCC-3'	Start site	
Notch-1a^{ATG}	5'-TTCACCAAGAAACGGTTCATAACTC-3'	Start site	248,249
Notch-1b^{ATG}	5'-ATGCATTCCTTCTTATGGATAGTCC-3'	Start site	
Notch-1b^{SPL}	5'-AATCTCAAACTGACCTCAAACCGAC-3'	intron28/exon29	189,250
Notch-5^{ATG}	5'-ATATCCAAAGGCTGTAATTCCCCAT-3'	Start site	189,251
Notch-6^{SPL}	5'-AGGTGAACACTTACTTCATGCCAAA-3'	exon7/intron7	189,251
PS-1^{ATG1}	5'-CCGGGATCATAGAAACAGCGGGAAC-3'	5' UTR	
PS-1^{ATG2}	5'-CATTCTGCACTAAATCAGCCATCGG-3'	Start site	
PS-2^{ATG}	5'-CTCTTCACTGTCTGAGGTATTCATG-3'	Start site	252
Prox1^{ATG1}	5'-TGGAACTGTTATTGTGCCATCTTCG-3'	5'UTR	
Prox1^{ATG2}	5'-ATGTGCTGTCATGGTCAGGCATCAC-3'	Start site	7
Prox1^{SPL}	5-CTTGGATGTGAAATTCGGAAGGTTA-3'	intron1/exon2	
Sox18^{ATG}	5-ATATTCATTCCAGCAAGACCAACAC-3'	Start site	253
Su(H)^{ATG}	5-CAAACTTCCCTGTCACAACAGGCGC-3'	Start site	190,254
Su(H)a^{ATG}	5-CGCCATCTTCACCAACTCTCTCTAA-3'	Start site	254
Su(H)b^{ATG}	5-CGCCATCTTCCACAAACTCTCACCA-3'	Start site	255
control MO	5'-CCTCTTACCTCAGTTACAATTTATA-3'	Standard control MO (Gene Tools)	

1.4 SCREENING METHODS FOR EVALUATION OF LYMPHATIC DEVELOPMENT AND FUNCTIONALITY

1.4.1 Scoring of TD and PL string formation in zebrafish

Live screening and quantification of thoracic duct (TD) formation was performed on anesthetized *Fli1:eGFP*^{y1} embryos (a few drops of 4 mg/ml Tricaine (Sigma) stock solution in 5 ml embryo water) at 6 dpf when this vessel was completely developed in control embryos. DAPT treated fish were screened at 4 dpf, since screening at 6 dpf was unfeasible because fish became opaque due to loss of trunk circulation and edema and many fish died between 4 and 6 dpf. For screening, images were acquired using Zeiss AxioVision 4.6 software on a Leica DM RBE fluorescence stereomicroscope equipped with a Zeiss AxioCam MrC5 digital camera (Carl Zeiss, Munich, Germany; Leica Microsystems, Wetzlar, Germany). For reasons of standardization and to correct for slight differences in embryo size, the percentage of thoracic duct formation was quantified by scoring its percentile presence in 10 consecutive somite segments in the trunk after the junction of DA and PCV (i.e. somites 5-15, see Figure 9). For screening of thoracic duct formation, only embryos with normal overall morphology and normal trunk circulation were included. All data are based on scorings of 33-185 embryos per condition, generated in at least 3 independent experiments. Because the penetrance of the lymphatic phenotype was variable, we also determined the fraction of embryos with severe, intermediate, subtle or no lymphatic defects for each treatment dose. Since parachordal lymphangiobast (PL) cells develop initially from lymphangiogenic secondary sprouts in a segmented pattern (Figure 3) [90], screening of PL string formation in the 10-somite segment of the trunk was performed in a similar manner at 52-56 hpf.

1.4.2 Functional assessment of the thoracic duct

For functional studies, anesthetized larvae were subcutaneously injected with 1 nl fluorescent dextran (2.5 mg/ml) into the muscle mass of the posterior trunk by using glass capillaries and a conventional microinjection setup [6]. Uptake of the dye in the thoracic duct was monitored. (this assay was performed in collaboration with the lab of S. Schulte-Merker, Utrecht, the Netherlands)

1.4.3 Time lapse imaging

Embryos were mounted in 0.5% low melting point agarose in a culture dish with a cover slip replacing the bottom. Imaging was performed with a Leica SP2 or SP5 confocal microscope using a 10x, 20x or 40x objective with digital zoom. Timelapse analysis was compiled using ImageJ software (http://rsb.info.nih.gov/ij/). Time points were recorded every 10 minutes for the stated time period. A heated stage maintained the embryos at approximately 28.3 °C.

1.4.4 Isolation of zebrafish LECs

1.4.4.1 LEC labeling

LEC labeling was performed on anesthetized (a few drops of 4 mg/ml Tricaine (Sigma) stock solution in 5 ml embryo water) *Fli1:eGFP^{y1}* zebrafish larvae of 3-4 weeks old. Tetramethyl-rhodamine-dextran (TRITC-dextran; molecular weight 2000 kDa) was injected intramuscularly into the tail somites. After 3-4 days the dye had been drained by the lymphatics and was taken up by the LECs through pinocytosis.

1.4.4.2 Fluorescence-associated cell sorting (FACS)

To obtain single cell supspensions, tails of at least 10 LEC labeled fish were collected, washed with distilled water, chopped, and incubated in 0.25% trypsin at 28°C until almost completely digested. The reaction was stopped by addition of 100μl fetal calf serum (FCS) to inhibit the trypsin and the cell suspension was loaded on top of a Cell strainer tube with blue filter cap (40μm; BD Biosciences) for filtration. After pelleting of the cells (5min, 200g), the cell suspension was washed with 1ml Dulbecco's PBS (dPBS) + 2% FCS and viable cells were counted using trypan blue exclusion. After pelleting, the cells were resuspended at a density of 10^6 cells/ml in FACS buffer (dPBS + 1% FCS – filtered).

Cells were sorted using a FACSAria (Becton Dickinson), taking care to exclude possible doublets or cell clusters. Non-injected GFP$^+$ *Fli1:GFP^{y1}* and TRITC-injected GFP$^-$ embryos were used as controls for proper compensation and gate setting. On average

25000 GFP^+TRITC^+ LECs and 50000 GFP^+TRITC^- BECs were sorted directly in lysis buffer from the RNeasy kit (Qiagen; RLT containing 1% β-mercaptoethanol).

2 XENOPUS LAEVIS EXPERIMENTS

2.1 HUSBANDRY & TRANSGENIC LINES

Sexually mature adult frogs were purchased from Nasco Biology (Fort Atkinson, WI) and housed in tanks with chlorine- and chloramines-free water. Frogs were kept in a 12h day/night cycle in a temperature-controlled environment. Transgenic *Flk1:eGFP Xenopus laevis* founderline frogs expressing green fluorescent protein (GFP) in blood and lymphatic vasculature were generated by restriction enzyme mediated insertion (REMI) transgenesis [256] using the promoter sequence of the *Xenopus laevis* gene VEGFR-2 (Flk1) driving eGFP ([257], manuscript in preparation). *Flk1:eGFP* transgenic embryos were obtained by natural mating of hormonally induced *Flk1:eGFP* females and wild-type males. Hormonal induction of ovulation was performed by pre-priming female adults with 200 units pregnant mare serum gonadotrophin (Folligon, Intervet) at least 4 days before priming. Priming was done at least 12 hours prior to egg collection, via a lymph sac injection with 500 units of human chorionic gonadotrophin (Chorulon, Intervet). The developing embryos were staged according to Nieuwkoop and Faber [258], kept in tadpole growth medium (0.1x Marc's Modified Ringer's (MMR) medium: prepared from a 10x MMR stock solution containing 1M NaCl, 20mM KCl, 10mM $MgSO_4$, 20mM $CaCl_2$, 50mM hepes, pH7.4) at 18°C until gastrulation was completed and from there on at 22°C [32].

2.2 *XENOPUS LAEVIS* COMPOUND INHIBITOR TREATMENTS

At developmental stage 26-28 [258] DAPT (Calbiochem) was added to the tank water at concentrations ranging from 25µM to 100µM. Control tadpoles were treated with the corresponding amount of DMSO. Compound/DMSO and growth medium were refreshed every day.

2.3 SCREENING METHODS FOR EVALUATION OF LYMPHATIC DEVELOPMENT AND FUNCTIONALITY

2.3.1 Live screening

At developmental stage 45 (5 dpf) the tadpoles were analyzed using a Zeiss SV11 stereomicroscope for heart beating, blood flow, lymph heart (LH) beating, and the presence or absence of edema and blood spots. In addition, the GFP positive blood and lymph vasculature in the trunk region was documented with a Zeiss Lumar V.12 fluorescence steromicroscope equipped with an AxioCam MrC5 (Zeiss) digital camera and controlled with AxioVision 4.6 software (Zeiss).

2.3.2 Functional assessment of the lymph vessels

Lymphangiography was performed as described [32] on anesthetized (in 0.02% 3-aminobenzoic acid ethyl ester (MS-222: Sigma, St. Louis, Missouri) dissolved in 0.1xMMR) stage 42-45 tadpoles placed on agarose gel in a petridish. The dye used was tetramethyl-rhodamine-dextran (TRITC-dextran; molecular weight 2000 kDa). Pictures were taken within 10 minutes of injection to avoid dye extravasation on a Zeiss Lumar V.12 fluorescence steromicroscope equipped with an AxioCam MrC5 (Zeiss) digital camera and controlled with AxioVision 4.6 software (Zeiss)

3 CELL CULTURE EXPERIMENTS

3.1 MAINTENANCE

Monkey kidney COS cells were grown in standard DMEM medium (Lonza, Invitrogen) supplemented with 10% FBS, 2 mM glutamin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Lonza). Human umbilical venous endothelial cells (HUVEC) cells (Lonza, Invitrogen, Merelbeke, Belgium), HUVEC/COS co-cultures, dermal (HMVEC-DLy) and lung

(HMVEC-LLy) lymphatic endothelial cells (Lonza, Invitrogen) were grown in EGM2-MV medium (Lonza, Invitrogen) at 37°C.

3.2 COCULTURE ASSAY

COS cells stably expressing full length human Dll4 (COS^{Dll4}) or expressing GFP (COS^{CTR}) were prepared using the retroviral constructs LZRSpBMN-DLL4 and LZRSpBMN-WT, respectively [259]. HUVECs were co-seeded with COS^{Dll4} or COS^{CTR} cells in 6-wells at a density of 200,000 cells each, grown for 24 hours and harvested for RNA analysis by quantitative RT-PCR using human gene-specific primers (Table 2).

3.3 PROLIFERATION ASSAY

Primary LEC (HMVEC-DLy or HMVEC-LLy; Lonza, Invitrogen) were starved overnight in EGM2 medium (Lonza, Invitrogen) containing 0.1% serum and no growth factors (starvation medium). The starved cells were seeded at 2,000 cells per well in 96-well microtiter plates, after which proliferation was induced with fully supplemented EGM2-MV medium with or without increasing concentrations of DAPT (20-60 μM). Proliferation was measured as the number of viable cells after further culturing for 48 hours, expressed in % of DMSO control. Viable cells were quantified using the Rapid Cell Proliferation assay (Calbiochem, San Diego, CA).

3.4 MIGRATION ASSAYS

3.4.1 Scratch wound migration assay

Confluent monolayers of LECs growing in 0.1% gelatin-coated wells of a 24-well plate were starved overnight, pretreated in starvation medium containing 30 μM DAPT or 0.3% DMSO (control), scratch wounded and photographed (T0). The cells were further incubated for 24 hrs and photographed again (T24). Migration distance (gap width at T0 minus gap width at T24; 10 measurements per wound at regular intervals along the wound) was

determined by image analysis using KS300 morphometry software, and is expressed relative to the control (DMSO).

3.4.2 Transwell migration assay in conditions of Notch inhibition

LECs were pretreated with DAPT (60 μM) or vehicle (DMSO) in starvation medium overnight, seeded at 30,000 cells per transwell on 0.1% gelatin-coated transwells in starvation medium with DAPT or DMSO, and cultured for 2 hours until adherence. Migration was induced by transferring the transwell insets into wells (bottom well) containing fully supplemented EGM2-MV medium with 100 ng recombinant human VEGF-C (Reliatech, TecoMedical NL, Nijkerk, the Netherlands), and DAPT or DMSO. Background migration was determined by including transwells with starvation medium in both the top and bottom well (baseline). After culturing for 16 hours, the non-migrated cells on the top side of the transwell filters were wiped off using PBS-soaked cotton swabs, and the transwells were fixed with 1% p-formaldehyde. The transwell filters were cut out and mounted upside-down on micrsocope slides with DAPI containing mounting medium. The filters were photographed under DAPI fluorescence at 20x magnification, and the nuclei were counted as a measure of migrated cells. Five transwells were prepared per condition and five optical fields were counted and averaged per transwell filter.

3.4.3 Transwell migration assay in conditions of Notch activation

LECs were starved overnight and seeded at 30,000 cells per transwell on transwell filters coated with BSA or with the extracellular domain of Dll4 (Dll4-ECD; R&D Systems Europe Ltd., Abingdon, UK) to activate the Notch pathway as described [206]. Further manipulation was as described above, using starvation medium in all top wells and fully supplemented medium containing VEGF-C in all bottom wells except for the baseline conditions. The filters were photographed under DAPI fluorescence at 10x magnification, Five transwells were prepared per condition and one cen tral optical field was counted per transwell filter.

3.5 BEAD SPROUTING ASSAY

HMVEC-DLy or HMVEC-LLy (Lonza, Invitrogen) cells were coated on Cytodex3 microcarriers (Amersham) by mixing beads and cells at a ratio 1/400 in full growth medium. This mixture was incubated for 4 hours at 37°C and mixed gently from time to time. Then the beads were transferred to a T25 cell culture flask and grown overnight in full growth medium. The next day the beads were washed 3 times with full growth medium and resuspended at a density of 800 cell-coated beads per ml in 0.4% methyl cellulose. One volume of bead solution was mixed with three volumes collagen solution (44% rat tail collagen (Roche), 1x DMEM, 0.033 N NaOH, 0.2M Hepes, 5% serum) and dispensed in a 96 well plate (100μl/well). After 30 minutes of solidification an equal volume (100μl) of full growth medium was added containing double concentrations of treatment reagents (DAPT or DMSO; final concentration of DAPT was 30 or 60μM). Sprouting was monitored over time and medium (supplemented with treatment agents) was refreshed every 2 to 3 days.

4 MOLECULAR BIOLOGY

4.1 RNA EXTRACTION

Total RNA from cultured cells was isolated using the RNeasy Kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA from zebrafish embryos was isolated by disrupting the tissue with a needle in Trizol reagent (Invitrogen, Belgium) and snap-freezing. Additional purification was done using the RNeasy MiniElute Cleanup Kit (Qiagen, Germany) according to the manufacturer's protocol. Total RNA from FACS sorted GFP^+TRITC^+ LECs and GFP^+TRITC^- BECs from LEC-labeled zebrafish larvae, sorted directly in lysis buffer (RNeasy kit; RLT containing 1% β-mercaptoethanol), was prepared using the RNeasy kit (Qiagen).

4.2 cDNA PREPARATION

cDNA from cultured cells and whole zebrafish embryos was prepared using the Quantitect Reverse Transcription kit (Qiagen, Germany). cDNA from FACS-sorted zebrafish cells was prepared using the SuperScriptIII kit from Ambion, which is better suited for small amounts of RNA.

4.3 QUANTITATIVE REAL-TIME PCR

Primer-sets and FAM™-TAMRA dye-labeled probes (Eurogentec, Belgium) were designed for zebrafish Her1 and β-actin and for human HES1, HEY1, HEY2, NRARP, VEGFR3, PROX-1, LYVE-1, EPHRINB2, SOX18, COUP-TFII, NEUROPILIN-1, CD31, VE-CADHERIN and ENDOGLIN (listed in Table 2). PCR reactions were carried out on a 7500 Fast Real-time PCR system (ABI, Germany). Each sample was analyzed in duplicate along with specific standards and no template controls. Amplifications were carried out using 2X TaqMan® Universal PCR Master Mix or 1x TaqMan® SYBR Green Universal Mix PCR reaction buffer (Applied Biosystems). Calculations of the initial mRNA copy numbers in each sample were made according to the cycle threshold (Ct) method. The copy numbers of the analyzed mRNAs were normalized using zebrafish or human β-actin mRNA levels.

TABLE 2: qRT-PCR PRIMER AND PROBE SEQUENCES

Zebrafish genes		
HER1	For	5'-CAT CGA GGG CCT GGA CAA-3'
	Rev	5'-AGG CGT AGC TAT TGA GAT GTG AAA-3'
	Probe	5'-FAM-ACA GAT CCC CTC CGC ATC CGT CTG-TAMRA-3'
NRARPA	For	5'-ATC ATG AGC CAG GCG GAT AT-3'
	Rev	5'-TTT ACC GCC TCC TGG AAG ACT-3'
	Probe	5'-FAM-ACG TGC TCG GCG CCG CA-TAMRA-3'
PROX1	For	5'-TCG TCC AAC CAC CTC AGT CA-3'
	Rev	5'-AGG GAC AAG GAC AAG CCT TCA-3'
	Probe	5'-FAM-ATC GTT CCA TCT CAC CAA CAC ACC CAG-TAMRA-3'
NOTCH1B	For	5'-AAC AAC CAA GAT CTT TCC CAT ATA CA-3'
	Rev	5'-GCT CTA GCC ATT CGC ATT GAC-3'
	Probe	5'-FAM-TTT GAT CCA TTG CCT CCA CGT CTC ACT-TAMRA-3'
DLL4	For	5'-CTT CAC CGG ACC CCT CTG T-3'
	Rev	5'-TGG AAG CGG TCT TGA GTT TCT C-3'
	Probe	5'-FAM-ATA CTA CGC CGT CAC AGC GCC CG-TAMRA-3'
β-ACTIN	For	5'-TGG TAT GGG ACA GAA AGA CAG CT-3'
	Rev	5'-TTG GGT ACT TCA GGG TCA GGA-3'
	Probe	5'-FAM-TCT TGC TCT GAG CCT CAT CAC CAA CG-TAMRA-3'
Human genes		
HES1	Hs00232622_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
HEY1	Hs00232618_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
HEY2	Hs00232622_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
NRARP	Hs01104102_s1 (Premade Taqman Gene expression assays, Applied Biosystems)	
VEGFR3	For	5'-TTC CTG GCT TCC CGA AAG T-3'
	Rev	5'-AGG CCA AAG TCA CAG ATC TTC AC-3'
	Probe	5'-FAM-ACC TGG CTG CTC GGA ACA TTC TGC-TAMRA-3'
PROX-1	For	5'-GTG CTT TGG CGA CGT CAT C-3'
	Rev	5'-TCA GTG GAA CTG GCC ATC TG-3'
	Probe	5'-FAM-TTC CGA ACC CCC TGG ACA CCT TTG-TAMRA-3'
LYVE-1	For	5'-CAA AGA TCC CAT ATT CAA CAC TCA A-3'
	Rev	5'-GGG ATG CCA CCG AGT AGG TA-3'
	Probe	5'-FAM-CTG CAA CAC AAA CAA CAG AAT TTA TTG TCA GTG ACA-TAMRA-3'
EPHRINB2	Hs00970627_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
SOX18	For	5'-AGA ACC CGG ACC TGC ACA-3' (Sybr Green qRT-PCR)
	Rev	5'-CAG CTC CTT CCA CGC TTT G-3'
COUP-TFII	Hs00819630_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
NEUROPILIN2	Hs00187290_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
NEUROPILIN1	For	5'-TGT GAA GTG GAA GCC CCT ACA-3'
	Rev	5'-GGC CTG –GTC GTC ATC ACA TT-3'
	Probe	5'-FAM-CCG ACC ACT CCC AAC GGG AAC TTG-TAMRA-3'
CD31	For	5'-TCT GCA CTG CAG GTA TTG ACA A-3' (Sybr Green qRT-PCR)
	Rev	5'-CTG ATC GAT TCG CAA CGG A-3'
VE-CADHERIN	Hs00174344_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
ENDOGLIN	Hs00164438_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
β-ACTIN	Hs99999903_m1 (Premade Taqman Gene expression assays, Applied Biosystems)	
β-ACTIN	For	5'-TGG CAC CAC ACC TTC TAC AAT G-3' (Sybr Green qRT-PCR)
	Rev	5'-TAG CAA CGT ACA TGG CTG GG-3'

5 HISTOLOGY

5.1 WHOLE-MOUNT IN SITU HYBRIDIZATION

5.1.1 In situ hybridization on zebrafish embryos

For whole-mount *in situ* hybridizaiton, dechorionated embryos were fixed overnight in 4% paraformaldehyde at 4°C and stored in methanol. *In situ* hybridization using antisense probes for EphrinB2a [260], Vegfr3 [261], Notch-1b, Notch-6, Dll4, Dab2 [90,253], Tbx20 [262,263], Cmlc2 or MyoD [264] was performed as described [264]. Briefly, embryos were rehydrated in graded methanol series and washed in PBST 0.1%, permeabilized in Proteinase K (10μg/ml in PBST), refixed for 20 minutes in 4% paraformaldehyde at room temperature and washed several times in PBST. Embryos were prehybridized for at least 1 hour in hybridization buffer (50% formamide, 5x SSC, 0.1% Tween20, 0.5 mg/ml Torula (yeast) RNA, 50μg/ml heparin, add 460 μl 1 M citric acid per 50 ml for pH 6.0) at 68°C and hybridized overnight at 68°C in hybridization solution containing probe (1/500 to 1/2000 dilution). The next day, embryos were washed 2x 30 minutes at 68°C in pre-warmed 50% formamide in 2x SSCT, 15 minutes in pre-warmed 2x SSCT, 2x 30 minutes in pre-warmed 0.2x SSCT and 5 minutes in MABT (100 mM maleic acid, 150 mM NaCl, pH7.5 containing 0.1% Tween20) at room temperature. Blocking was performed for at least 1 hour in block solution (2% DIG-block (Roche) in MABT) and embryos were incubated overnight at 4°C in anti-DIG-AP antibody (1/2000 in blocking solution). Finally, the embryos were washed 4x 15 minutes in MABT at RT and stained in BM purple staining substrate (Roche). Staining was monitored over time and stopped by washing the embryos in PBST several times and refixing for several hours in 4% paraformaldehyde. Embryos were stored in 70% glycerol. Stained embryos were paraffin- or plastic-embedded, sectioned and counterstained with nuclear fast red.

5.1.2 In situ hybridization on Xenopus embryos

In situ hybridization on *Xenopus* embryos was performed according to Harland's protocol [265]. The probe used was a DIG-labeled (DIG RNA labeling kit, Roche) antisense RNA fragment targeted against xProx1 (nt +190 till +1139 in reference to the ATG start codon). As a negative control the sense probe was used. Briefly, embryos were fixed overnight at

the desired stage in Memfa fixative (3.7% paraformaldehyde, 100mM MOPS, 2mM EGTA, 1mM $MgSO_4$ in DEPC treated MQ water) at 4°C and rinsed in EtOH. Fixed embryos can be stored in absolute EtOH at -20°C. Embryos were rehydrated and permeabilized in 10μg/ml Proteinase K (Roche) in PBST 0.1% for at least 10 minutes, depending on the developmental stage. To reduce non-specific binding of the probe, embryos were treated with acetic anhydride in 0.1M triethanolamine. After a short postfixation in 3.5% formaldehyde in PBST 0.1%, the embryos were prehybridized at least 10 minutes at 60°C in 25% hybridization buffer (50% formamide, 5x SSC, 1mg/ml Torula RNA, 100μg/ml heparin, 1x Denhardt's, 0.1% Tween20, 5mM EDTA) in PBST 0.1%. Hybridization was performed overnight at 60°C in hybridization buffer containing 0.5 μg/ml of probe. The morning after, the embryos were blocked in MAB-BR (100mM maleic acid, 150mM NaCl, pH7.5 with 2% blocking reagent) after which they were incubated for at least 4 hours in MAB-BR supplemented with 20% heat-inactivated sheep serum containing a 1/2000 dilution of anti-DIG-AP antibody. After extensive washes in MAB, staining was done in darkness with BM purple substrate (Roche). Staining was monitored over time and the chromogenic reaction was stopped by washing the embryos with PBST and postfixation in Memfa fixative overnight at 4°C. Afterwards, embryos were bleached in bleaching solution (0.5% SSC, 5% formamide, 1.5% H_20_2), cleared in 100% EtOH, rehydrated and stored in 100% glycerol.

5.2 *IN SITU* HYBRIDIZATION ON CRYOSECTIONS OF ZEBRAFISH EMBRYOS

Zebrafish embryos were fixed overnight in 4% paraformaldehyde at 4°C and subsequently kept overnight in 20% sucrose containing a drop of eosine. Embryos were embedded in tissue-freezing medium (Neg -50, Richard Allan Scientific), snap frozen and 10μm sections were made on a cryo-microtome (Leica CM3000).

The slides were washed 4 times for 10 minutes in PBST 0.3%, pre-hybridized in hybridization buffer (1mg/ml Torula RNA, 50% deionized formamide, 1x salt (for 100mL: 11.4g NaCl, 1.4g Tris-HCl, 0.134g Tris Base, 0.69g $NaH_2PO_4.2H_2O$, 0.71g Na_2HPO_4, 10mL EDTA, H_2O), 50% dextran sulphate, 1x Denhardts) for at least one hour at 68°C and incubated overnight with probe diluted 1/200 – 1/1000 in hybridization buffer. The next day the slides were washed 1x 15 minutes and 2x 30 minutes in washing buffer (50%

formaldehyde, 1x SSC, 0.1% Tween20) and 2x 30 minutes in MABT 0.1%. Subsequently, the slides were blocked in 2% DIG-block (Roche) in MABT and incubated overnight in anti-DIG-AP antibody diluted 1/2000 in DIG-block. Finally, the slides were washed 4x 20 minutes in MABT 0.1% and incubated for several days in BM purple staining substrate (Roche).

5.3 IMMUNOSTAINING

Immunostaining was performed using standard protocols. Antibodies used for Prox1 immunostaining in zebrafish were from Abcam, Reliatech, AngioBio and Chemicon [7,266-268].

6 STATISTICAL ANALYSIS

Absolute values were used to calculate means and SEMs. Each gene-specific morpholino (or compound dose) was always compared to the control morpholino or vehicle in every single experiment. Pairwise comparisons were performed between the different doses by two-sided t-test unless otherwise specified. The asterisks on the figures represent the treatment difference at a significance level of $P<0.05$. To determine the penetrance of the phenotype, we counted the number of zebrafish embryos, exhibiting the different severities of morphant phenotype. Chi-square analysis was used to determine whether the severity distribution differed between treatment groups.

Chapter IV

RESULTS

1 INHIBITION OF NOTCH SIGNALING IMPAIRS LYMPHATIC DEVELOPMENT IN ZEBRAFISH EMBRYOS AND TADPOLES

1.1 GENERAL INHIBITION OF THE NOTCH PATHWAY BLOCKS LYMPHANGIOGENESIS IN AQUATIC ANIMAL MODELS

In a first attempt to explore a role for Notch signaling in lymphatic development, we took advantage of the fact that activation of the Notch receptor, which requires proteolytic cleavage by the γ-secretase complex to release the intracellular domain, can be selectively blocked by chemical γ-secretase inhibitors [269]. A well known γ-secretase inhibitor, that has been previously used to block Notch signaling in aquatic models is *N*-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) [188]. DAPT was retracted from a screening of compounds that were tested for their ability to inhibit amyloid β (Aβ) production, which is formed by γ-secretase mediated proteolytic carboxy-terminal cleavage of the amyloid precursor protein (APP) [270]. Overproduction of the fibrillogenic 42 amino acid Aβ-variant (Aβ42) is the primary cause of plaque formation in the brain during Alzheimer's disease [271]. The primary mechanism of action of DAPT is via functional inhibition of the γ-secretase complex [270], more specifically by targeting the catalytic subunit, the aspartyl protease presenilin [272]. Unlike other γ-secretase inhibitors, DAPT has remarkably low activity against structurally related presinilin-like aspartyl proteases, like Signal Peptide Peptidase (SPP) [273]. Therefore, DAPT is considered to be the most specific γ-secretase inhibitor [274].

1.1.1 Zebrafish

Embryos of the transgenic *Fli1:eGFP^{y1}* zebrafish line, which expresses GFP in blood and lymph vessels [6,7,87], were exposed to the γ-secretase inhibitor DAPT at early stages. At 4 dpf the formation of the thoracic duct (TD), the first and major axial lymph vessel in the zebrafish trunk, was analyzed [6,7] (for details on the formation and developmental stages of the lymph and blood vessels, see Introduction p. 19 and Figure 3).

Since Notch signaling is involved in somitogenesis and angiogenesis [275], and since the TD originates from the PCV [7,90], inhibition of Notch could impair lymphatic development indirectly by affecting vascular development or the intersomitic migration pathways of PL

cells. To avoid such secondary effects, and to study more selectively a primary effect of γ-secretase inhibition on lymphangiogenesis, we first determined in pilot experiments a tolerable concentration range of DAPT (6.25-25 µM) and analyzed at which developmental stage the embryos were best exposed to DAPT. At these concentrations DAPT did not affect arterial-venous differentiation of the DA and PCV as the expression pattern of arterial and venous marker genes (*EphrinB2a* and *VEGFR-3*, respectively) was normal by whole mount ISH staining (Figure 14Q-R). Consistent with previous findings [172,189,190], DAPT caused impaired somite development, axial vessel malformations and hyperbranching of ISVs when administered at early developmental stages. Major vascular and morphological defects could be avoided by using a concentration of maximally 25µM DAPT and initiation of the treatment at later developmental stages. Based on these developmental criteria we initially selected 43 hours post-fertilization (hpf) for our studies. Although at this stage lymphangiogenic sprouting from the PCV has already started and PL formation is ongoing (see Figure 3), we observed impaired formation of the TD.

TD formation was scored by measuring the length over which the TD had formed by 4 dpf, when the lymph vessel was nearly completely established in control embryos. Screening of DAPT treated embryos at 6 dpf, when TD formation is completed in control embryos, was not feasible because DAPT-treated embryos became opaque due to loss of trunk circulation and edema and many fish died between 4 and 6 dpf. For reasons of standardization and correction for slight differences in embryonic size, the data were expressed as % of the length of the fragment of the trunk analyzed, i.e. the 10 consecutive somite segments in the trunk after the junction of DA and PCV (somites 5-15; Figure 9). To further avoid that the documented defects reflected secondary effects of vascular malformation on lymphatic development, only embryos with normal axial blood flow were screened for TD formation in all further experiments. Treatment of embryos with DAPT dose-dependently lowered mRNA transcript levels of the Notch-regulated target genes zHer1 and zNrarpA, indicating that Notch receptor activity was effectively inhibited (Figure 10).

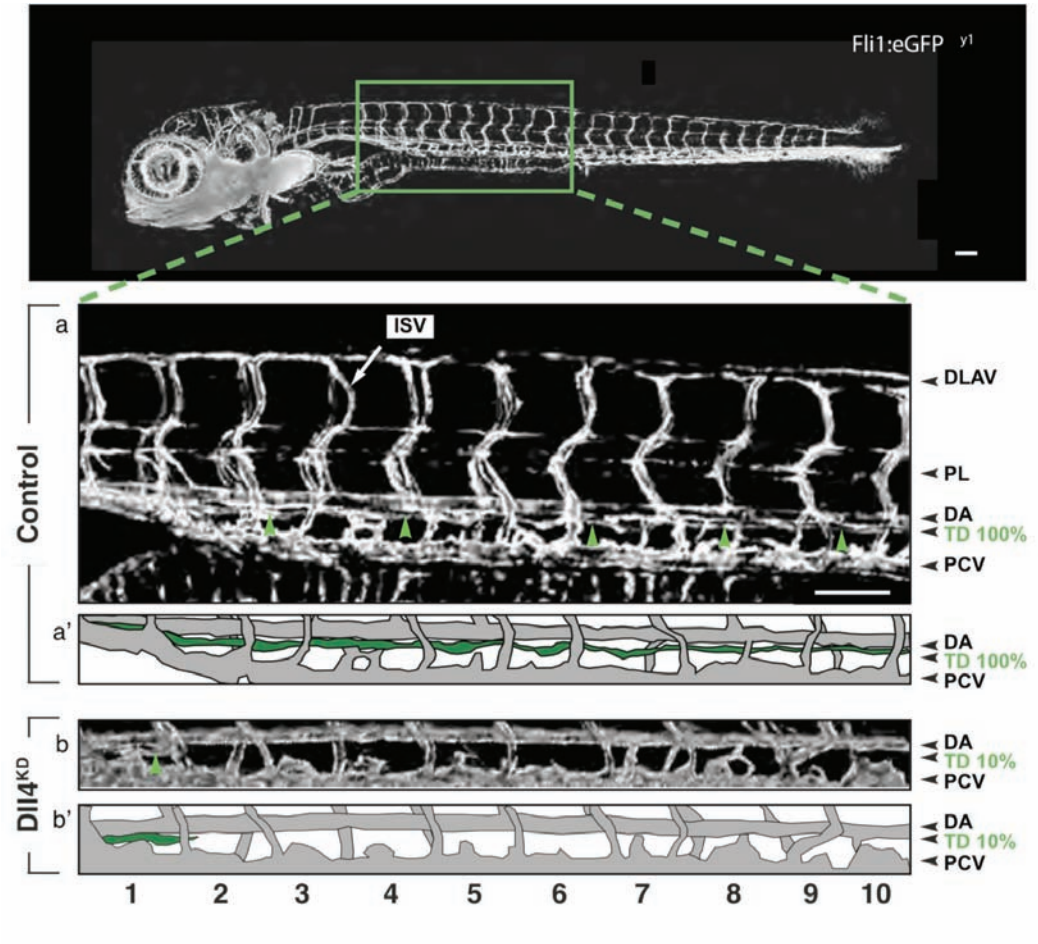

Figure 9: Method of TD quantification. *DA, dorsal aorta; DLAV, dorsal longitudinal anastomosing vessels; ISV, intersomitic vessel; PCV, posterial cardinal vein; PL, parachordal lymphangioblast string; TD, thoracic duct. TD formation was quantified by measuring the length over which it formed in 10 consecutive somite segments (i.e. somites 5-15; demarcated by the green rectangle). Confocal images of control and morphant Fli:eGFP^{y1} embryos are depicted in the insets. Inset a: in the control embryo, a continuous TD formed over all 10 somite segments (100% TD formation; green arrowheads). Inset b: severely morphant embryo, in which the TD formed over only 10% (green arrowhead). Inset a' and b': schematic redrawing of the DA, TD and PCV in the embryo shown in inset a and b, respectively, with the TD or TD segment marked in green. Scale bars: 100 µm.*

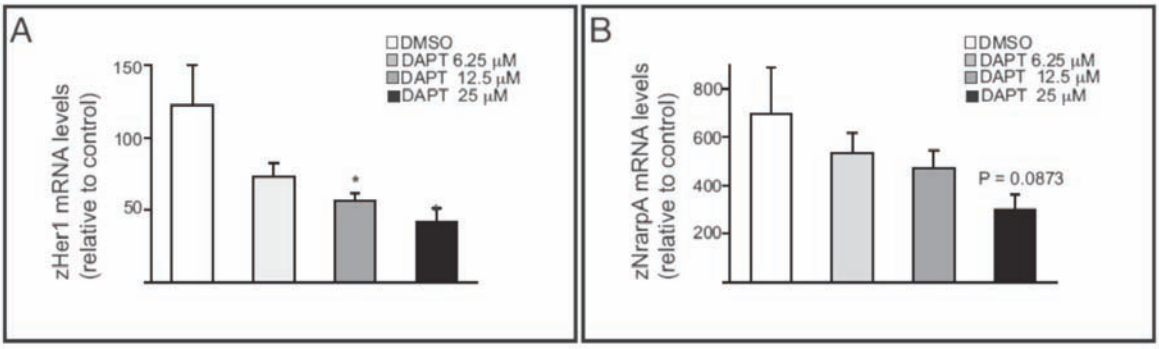

Figure 10: Verification of Notch pathway silencing. *Quantitative RT-PCR analysis of the Notch target genes zHer1 and zNrarpA on cDNA of DAPT treated zebrafish embryos. Data are shown as number of mRNA copies per 10^5 copies of zβ-actin. RNA was extracted from 3 dpf zebrafish embryos treated from 43 hpf onwards with increasing concentrations of DAPT or with DMSO (0.25%, open bar). Expression of zHer1* ***(A)*** *and zNrarpA* ***(B)*** *were reduced dose-dependently in DAPT-treated zebrafish embryos indicating that the Notch signaling pathway is effectively inhibited. Data are an average of 3-5 independent experiments. Error bars represent SEM. *, P<0.05.*

Embryos exposed to 25 µM DAPT at 43 hpf had a curved trunk and regularly exhibited hemorrhages in the head (Figure 11A-B), characteristics previously observed after Notch pathway knockdown [170,172,190,276]. Fluorescent analysis of live *Fli1:eGFP^{y1}* embryos at 4 dpf showed normal development of the trunk blood vasculature, but a dose-dependent impairment of TD formation (Figure 11C-D). At 25 µM DAPT, 53% of the analyzed embryos completely lacked a TD (*N* = 102), another 39% of embryos formed short TD fragments over 10-30% of its normal length, only 8% formed a relatively large TD fragment over 30-90% of the normal length and no embryos were observed with a normal TD (Figure 11E). The DAPT effect was dose-dependent and specific, as treatment with control vehicle (0.25% DMSO) did not adversely affect TD formation (Figure 11E). Interestingly, the finding that lymphatic defects were observed at lower DAPT concentrations than needed to induce vascular malformations, suggests a higher sensitivity of lymphangiogenesis over angiogenesis for Notch signaling.

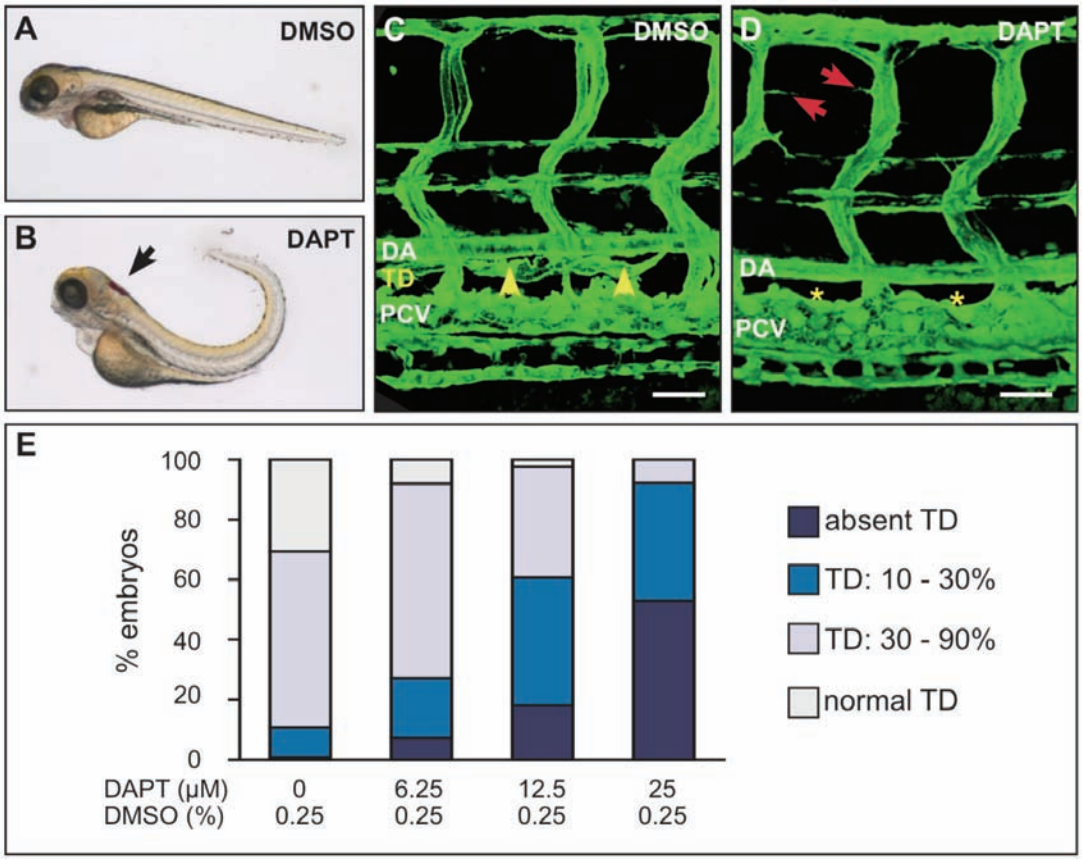

Figure 11: DAPT treatment of zebrafish embryos impairs thoracic duct development. (A,B) *Bright field pictures of live embryos at 4 dpf, showing curved body and hemorrhages in the head (arrow) in DAPT (25 μM) treated zebrafish (B) as compared to control DMSO (0.25%) treated fish (A).* ***(C-D)*** *Confocal images of GFP^+ vessels in the trunk of 4 dpf $Tg(Fli1:EGFP)^{y1}$ zebrafish embryos, revealing the formation of a normal lymphatic thoracic duct (TD) in the control embryo (C; yellow arrowheads) but not in the DAPT treated embryo (D; yellow asterisks). Small sprouts on the ISVs can be observed in the upper part of the DAPT treated embryos (D; red arrows).* ***(E)*** *Quantitative analysis of TD defects after treatment with different doses of DAPT or control DMSO.*

1.1.2 Xenopus laevis

In order to confirm our findings in the zebrafish model in a second, independent animal model, we also studied the role of Notch signaling in Xenopus tadpoles *, which develop an elaborate lymphatic system and were previously validated as a genetic model to study lymphangiogenesis [32]. More particularly, we used tadpoles of a newly developed transgenic *Xenopus laevis* line, i.e. *Flk1:eGFP*, that expresses eGFP under control of the frog VEGFR-2/Flk1 promotor in both blood and lymphatic vessels ([257], manuscript in preparation).

To block the Notch pathway, tadpoles were treated with DAPT (25-50 µM; at higher doses, the compound precipitated) and the effect on vascular and lymphatic development was analyzed by live screening under bright light microscopy, by whole-mount *in situ* hybridization for *xProx-1* to visualize the caudal lymph vessels at stage 42, by fluorescence microscopy of GFP^+ vessels at later stages, and by functional lymphangiography. We focused our analysis on the formation of the ventral (VCLV) and dorsal caudal lymph vessel (DCLV) in the tail. These major trunk vessels are formed primarily by LECs that transdifferentiate from the BECs of the PCV and migrate ventrally or dorsally to coalesce at the ventral margin or dorsal roof, grow out laterally, and interconnect to form a continuous VCLV or DCLV [32]. To minimize effects on general development, which might secondarily affect vascular development, tadpoles were exposed to DAPT from stage 26-28 onwards (2 dpf), i.e. at the initiation of the first lymphatic structures [32].

Live screening by bright field microscopy at stage 45 (corresponding to 5 dpf) revealed that DAPT induced edema around the cardiac region, blood spots in the brain, ventralized body shape and eye deformity (Figure 12A-B), resembling features of the phenotype after DAPT treatment in zebrafish (Figure 11A-B). At 25 or 50 µM DAPT, 41% and 54% of tadpoles exhibited some abnormalities in blood flow (usually slowed down, or occasionally absent in the dorsal longitudinal anastomosing vessel, DLAV). By fluorescence microscopy, the overall architecture of the GFP^+ blood vasculature appeared, however, largely normal, suggesting that the blood vessels formed, but had a smaller lumen (as observed previously

* Studies in the *Xenopus* model were performed with the collaboration of Wouter Vandevelde and Annelii Ny (Vesalius Research Center, Leuven)

in mice upon inhibition or loss of Notch signaling [161,162,164]) and, therefore, were hypoperfused.

In situ hybridization for *xProx-1* at stage 42 (representing the oldest stage at which whole-mount in situ hybridizations can be reliably performed in tadpoles) revealed lymphatic defects in DAPT-treated tadpoles. In control embryos, Prox-1$^+$ LECs are aligned in a continuous DCLV and VCLV, nearly over the entire length of the trunk (Figure 12C). By contrast, in DAPT-treated tadpoles (25-50 µM), the DCLV was only partially formed and consisted of isolated clusters of Prox-1$^+$ LECs, discontinuously and non-homogeneously distributed over the length of the DCLV (Figure 12D). While fluorescence imaging of the *Flk1:eGFP* tadpoles revealed close alignment of the DCLV and DLAV in control tadpoles (Figure 12E), partial separation of the DCLV and DLAV was frequently observed in DAPT-treated tadpoles (Figure 12F), which may signify (microscopic) lymphedema. The VCLV appeared largely normal, at least by this analysis (Figure 12G-H) (see also below). Quantification of the lymphatic defects at stage 45 confirmed a disorganized, irregular and fragmented DCLV in 76% and 83% of tadpoles treated with 25 or 50 µM DAPT, respectively, while control tadpoles always developed a normal DCLV (Figure 12I). The VCLV was usually present at both DAPT concentrations tested (Figure 12G-H); as shown below, this lymph vessel was, however, severely dysfunctional at the highest DAPT concentration. Since the VCLV develops at a slightly earlier developmental stage than the DCLV [32] (and only a few hours after addition of DAPT), we speculate that its formation might not have been impaired as severely as that of the DCLV. With respect to formation of the latter vessel, DAPT had more time to penetrate into the tadpole and reach higher (sufficient) levels to impair its formation.

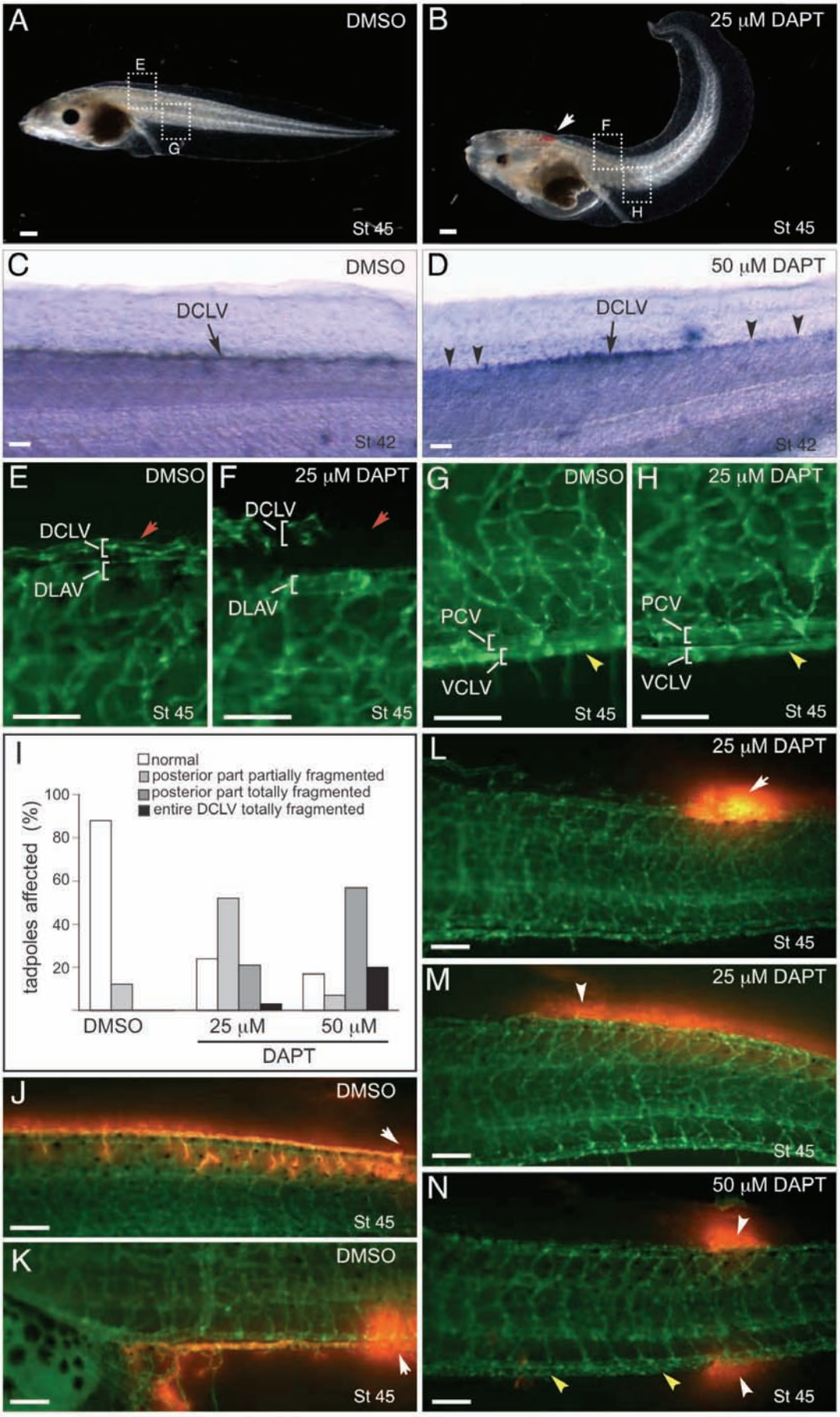
A
DMSO
E
G
St 45
B
25 μM DAPT
F
H
St 45
C
DMSO
DCLV
St 42
D
50 μM DAPT
DCLV
St 42
E
DMSO
DCLV
DLAV
St 45
F
25 μM DAPT
DCLV
DLAV
St 45
G
DMSO
PCV
VCLV
St 45
H
25 μM DAPT
PCV
VCLV
St 45
I
normal
posterior part partially fragmented
posterior part totally fragmented
entire DCLV totally fragmented
tadpoles affected (%)
100
80
60
40
20
DMSO
25 μM
50 μM
DAPT
L
25 μM DAPT
St 45
M
25 μM DAPT
St 45
N
50 μM DAPT
St 45
J
DMSO
St 45
K
DMSO
St 45

Figure 12: DAPT impairs lymphatic vessel formation in Xenopus tadpoles. ***(A,B)*** *Bright field pictures of live embryos at stage 45 (5 dpf), showing curved body, hemorrhages in the head (arrow) and malformed eyes in a DAPT (25 μM) treated tadpole (B) as compared to a control DMSO (1 %) treated tadpole (A).* ***(C,D)*** *Whole-mount* in situ *hybridization for xProx-1, revealing incomplete formation of the DCLV (arrowheads) in stage 42 tadpoles treated with 50 μM DAPT (D) as compared to normal formation of the DCLV in control tadpoles (C).* ***(E-H)*** *Fluorescent analysis of blood and lymph vessels in transgenic* Flk1:eGFP *tadpoles at stage 45. Areas in the trunk corresponding to the boxed areas in panels A or B are shown. In DMSO treated tadpoles, both the DCLV (E) and the VCLV (G) developed normally. Treatment with 25 μM DAPT resulted in a hypoplastic, disorganized and discontinuous DCLV (F), while the VCLV (H) had a grossly normal appearance. The partial separation of the DCLV and DLAV (F) may signify (microscopic) lymphedema.* ***(I)*** *A dose-dependent increase in the severity of the DCLV phenotype could be observed. The graph represents the percentage of tadpoles scored according to the following categories of DCLV defects (n=30-33): normal development of the DCLV (open bars); normal development of the anterior part of the DCLV, but partial fragmentation of the posterior part (light gray bars); normal development of the anterior part of the DCLV, but fragmentation of the entire posterior part (dark gray bars); total fragmentation of the entire DCLV (black bars).* ***(J-N)*** *Lymphangiography of stage 45 tadpoles revealing dysfunction of the DCLV and VCLV after DAPT treatment (L-N) in contrast to normal controls (J,K). Panel L shows a representative tadpole treated with 25 μM DAPT, displaying complete failure of drainage of the injected red fluorescent dye; panel M shows a tadpole in which the dye was initially taken up by backflow (i.e. in the direction away from the heart) in the DCLV but instantaneously diffused out, indicating leakiness of the vessel. Panel N shows a representative tadpole treated with 50 μM DAPT, with dysfunction of the DCLV and - despite its normal appearance (yellow arrows) - the VCLV. White arrowheads in panels J-N indicate injection site of the fluorescent dye. DCLV, dorsal caudal lymph vessel; DLAV, dorsal longitudinal anastomosing vessel; PCV, posterior cardinal vein; VCLV, ventral caudal lymph vessel. Scale bar represents 200 μm in panels A and B; 100 μm in panels C,D, J-N; 50 μm in E-H. In all panels, the tadpoles are oriented with their heads and lymph hearts facing left.*

Recent publications on the role of Notch in angiogenesis stressed the importance of determining the perfusion, and not simply the presence, of blood vessels, because morphologically detectable vessels may be hypoperfused and not functional [197]. We therefore determined the functional capacity of the lymph vessels to drain an injected red dye by lymphangiography at stage 45. When scoring the DCLV in ten tadpoles treated with 25 μM DAPT, the dye was either not drained at all (4 tadpoles; Figure 12L), drained only over a very short distance (5 tadpoles; not shown), or was initially absorbed, but then drained in the reverse orientation and rapidly leaking out, indicating that this lymph vessel

was dysfunctional (Figure 12M). At 50 µM DAPT, the dye was not drained at all in the majority of embryos tested (Figure 12N). A similar analysis revealed that the VCLV, despite its apparent normal morphological appearance, failed to drain the dye in all tadpoles treated with 50 µM DAPT, suggesting that its lumen was hypoplastic (Figure 12N). In all control tadpoles, both the DCLV and VCLV were fully functional (Figure 12J-K). Thus, similar to zebrafish, general inhibition of the Notch pathway impairs lymphatic development in Xenopus tadpoles.

1.2 ANALYSIS OF THE ROLE OF NOTCH PATHWAY COMPONENTS IN ZEBRAFISH BY SPECIFIC MORPHOLINO KNOCKDOWN

Since pharmacological inhibition of the Notch pathway by DAPT blocks *all* canonical Notch signaling pathways and additionally affects other γ-secretase mediated pathways (see higher), we wanted to analyze more specifically which Notch ligand/receptor pairs and downstream regulators are required for lymphatic development. Small animal models like zebrafish and *Xenopus* allow easy targeted knockdown of gene expression by injection of fertilized eggs with morpholino antisense oligonucleotides. For this project all further experiments were focused on the zebrafish model.

Morpholino oligomers are used as an antisense technology tool to specifically knock down the expression of a targeted gene. Morpholinos are small (~25 bases) antisense RNA fragments with an altered backbone structure, where nucleic acid bases are bound to morpholine rings instead of deoxyribose rings, and linked through phosphorodiamidate groups instead of phosphates. This structure is very stable and enables a strong binding of morpholinos to their target sequence. Morpholinos do not induce degradation of their RNA targets but act via ‘steric blocking’ of either the translation-initiation site (ATG-binding morpholinos and 5’-UTR morpholinos binding upstream but close to the ATG) or a splice-site (splice-blocking morpholinos).

In order to analyze the contribution of the different Notch ligands, receptors, and regulators or downstream effectors to lymphatic development, we specifically knocked down the single genes in zebrafish embryos and analyzed TD formation.

As Notch-family members have been implicated in angiogenesis [183], we used submaximal doses of all morpholinos to minimize secondary effects of vascular malformations on lymphatic development (referred to as “incomplete silencing” and “Notch hypomorphants”). In addition, only morphant embryos with a normal size, trunk circulation and blood flow, and without developmental delay, tissue malformations, general edema or toxic defects (not shown) were included in the screening.

1.2.1 Ligands: Knockdown of Dll4 impairs development of the thoracic duct

Dll4 knockdown ($Dll4^{KD}$) inhibited the formation of the TD. Upon injection of a morpholino affecting Dll4 mRNA splicing ($Dll4^{SPL}$; 10 ng), the TD failed to form at all by 6 dpf in up to 52% of the morphant embryos, indicating that lymphatic development was completely aborted (Figure 13 A-C). In another 27% of $Dll4^{SPL}$ embryos, the TD formed over only 10 to 30% of its normal length, while in another 15% of morphant embryos, the TD formed over 30 to 90% (Figure 13C). As explained above, initiation of TD formation occurs via sprouting of lymphangiogenic secondary sprouts at discrete locations alongside the PCV, i.e. on average one per two unilateral somite segments. Thus, if the TD formed over only 20% of its entire length, then TD development was in fact completely aborted in eight out of ten somites analyzed. We speculate that the variably penetrant lymphatic phenotypes and spectrum of defects in the Notch hypomorphants is due to a combination of reasons, including the use of a submaximal dose of morpholino (incomplete silencing), technical limitations of injecting an identical dose of morpholino, genetic differences of the morphant embryos analyzed (outbred background; we have observed 10-fold variable expression levels for Dll4 in single zebrafish embryos (not shown)), uneven dispersion of morpholinos upon daughter cell division, and variable timing of venous/lymphatic secondary sprout formation along the PCV (occurring within a time window of 30 to 50 hpf). As a result, achieving the necessary degree of silencing below the critical biological threshold at the distinct sprouting locations along the PCV becomes stochastic in such experimental conditions. The observed phenotypic defects were dose-dependent, but for reasons of brevity, only the highest dose is shown.

Follow-up studies at later stages (up to 12 dpf) revealed that, in embryos with intermediate defects, the few LECs that did arise and formed parts of the TD, were unable to reconstitute the entire TD and could not compensate for the lymphatic failure in nearby somites (not shown). Dll4^{KD} embryos without TD at 6 dpf also failed to form a TD, even partially, at later stages (Figure 13D,E), indicating that lymphatic development was not simply delayed, but aborted.

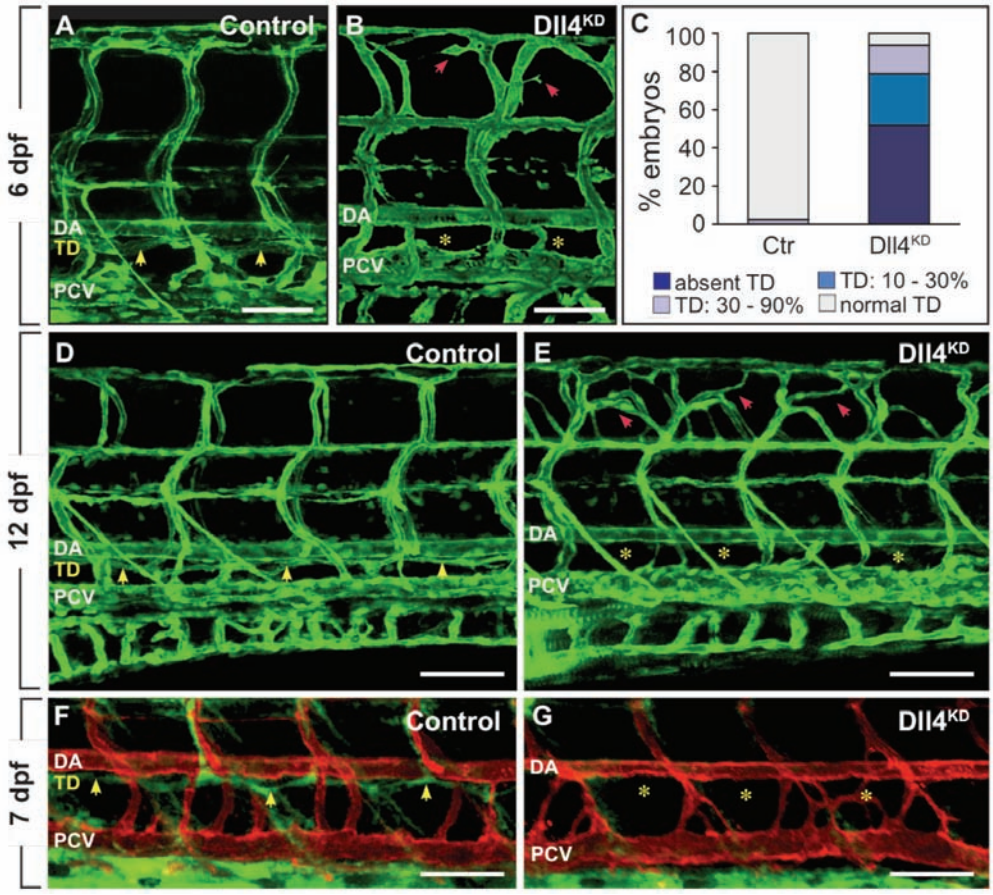

Figure 13: Dll4 knockdown impairs TD formation. A-B, D-E, *Confocal images of GFP^{+} vessels in the trunk of 6-dpf (A,B) or 12 dpf (D,E)* Fli1:eGFP^{y1} *embryos. The control embryos have a normal TD (yellow arrowheads in A and D), while the TD is entirely absent in the Dll4^{KD} (B, E). Yellow asterisks denote absence of the TD; red arrowheads indicate hyperbranching of the upper part of the ISVs.* ***C,*** *Bar graphs, representing the percentage of embryos that develop a normal TD (grey), a TD over 30-90% (lilac) or over 10-30% (blue) of its normal length, or completely lack any TD (dark blue) in the indicated fraction of control embryos (*N=*122) or Dll4^{KD} embryos (*N=*80; 10 ng Dll4^{SPL};* P<*0.001* versus *control by Chi-Square test).* ***F,G,*** *Lymphangiography in the trunk of 7-dpf* kdr-l:mCherryRed *embryos revealed normal uptake and drainage of a green dye by the TD in the control embryo (yellow arrowheads in F), but not in the Dll4^{KD} embryo (yellow asterisks in G).Scale bars: 50 μm.*

Lymphangiography in 7-dpf *kdr-l:mCherryRed* Dll4^{KD} embryos [#] (in which the *kdr-like* promoter drives expression of mCherryRed in the blood vessels while lymph vessels are not labeled) revealed no drainage of green fluorescent dye in the region where the TD normally forms, confirming that the lack of a GFP^{+} TD in *Fli1:eGFP^{y1}* Dll4^{KD} embryos was not due to reduced expression of this marker, but to actual absence of the vessel itself (Figure 13 F,G). Also, partial TD fragments did not take up injected dye, indicating that they are not functional (not shown).

The lymphatic defects in Dll4^{KD} embryos were specific, as no overt changes in the formation and differentiation of the DA and PCV were noticeable (Figure 14A-H). Consistent with previous reports [92,189], some hyperbranching of the primary ISVs was detected in Dll4^{KD} hypomorphant embryos, but to a variable degree and in only 20% of Dll4^{KD} embryos (red arrowheads in Figure 13B,E); hence, TD defects were detected in the majority of Dll4^{KD} embryos that did not exhibit any signs of ISV hyperbranching. Also, no abnormalities in heart or somite development were observed (as analyzed by *in situ* staining for Cmlc2 (Cardiac Myosin Light Chain 2) and MyoD (Myogenic Differentiation 1), respectively; not shown).

Similar TD defects were obtained with a second morpholino, targeting the ATG region of Dll4 (Dll4^{ATG}), thereby confirming the specificity of the Dll4^{KD} phenotype (not shown). Silencing of the other Notch ligands DeltaA-D, Jagged-1a/b or Jagged-2 did not induce lymphatic defects (not shown).

[#] experiments performed by the Schulte-Merker lab (Hubrecht Institute-KNAW and University Medical Center, Utrecht, the Netherlands)

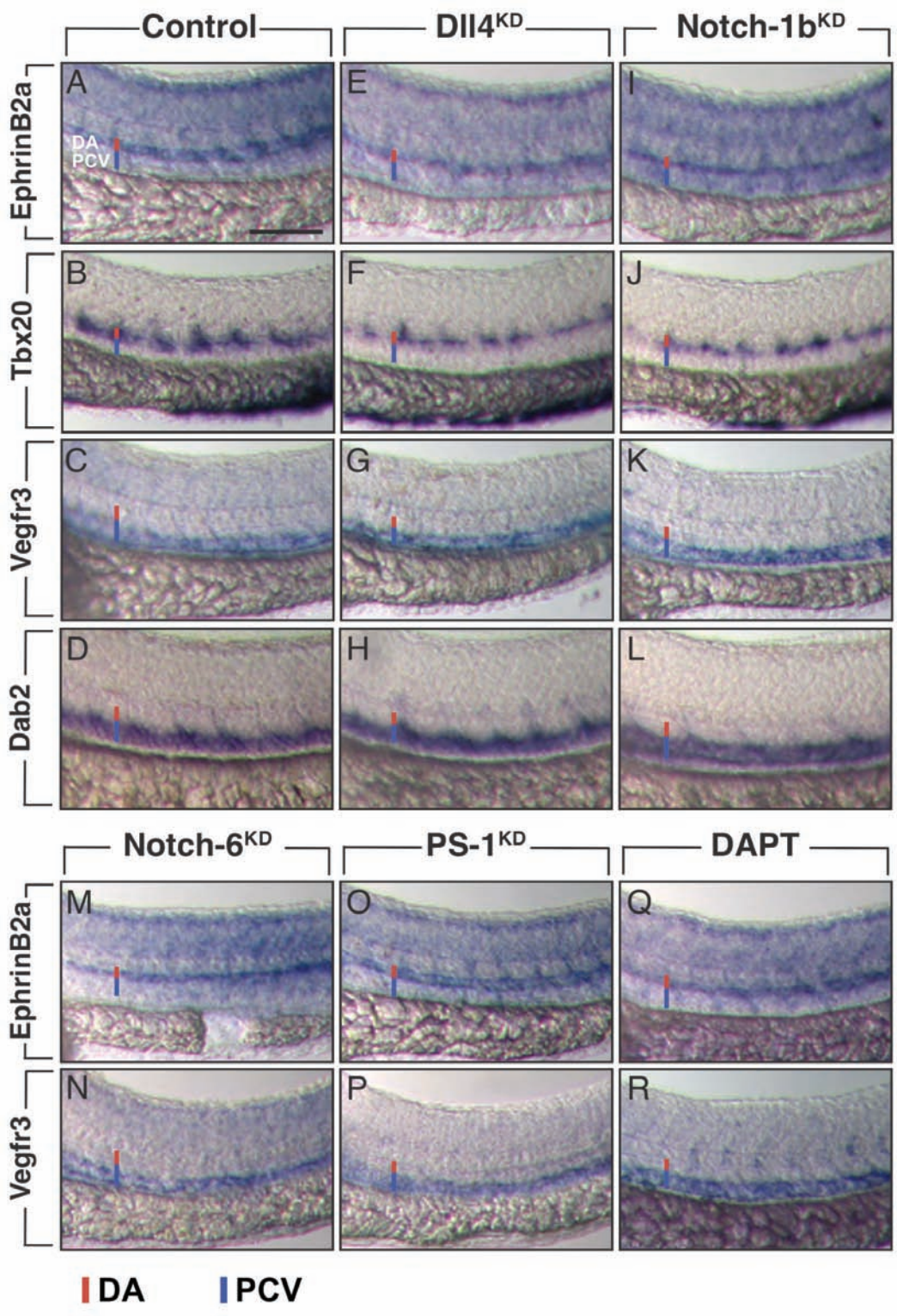
Control
Dll4KD
Notch-1bKD
EphrinB2a
Tbx20
Vegfr3
Dab2
A
B
C
D
E
F
G
H
I
J
K
L
DA
PCV
Notch-6KD
PS-1KD
DAPT
M
N
O
P
Q
R
DA
PCV

Figure 14: Normal arterial-venous differentiation after inhibition of Notch signaling. A-R, *Arterial-venous differentiation of the large axial vessels was evaluated upon incomplete silencing of the components of the Dll4/Notch signaling pathway that were shown in this study to affect lymphatic development. Therefore, whole-mount embryos were* in situ *stained for arterial (*EphrinB2a; Tbx20 [262,263]*) and venous (*Vegfr3; Dab2 [253,277]*) markers in control embryos (A-D) and in Dll4^{KD} (10 ng Dll4^{SPL}; E-H), Notch-1b^{KD} (15 ng Notch-1b^{SPL}; I-L), Notch-6 (15 ng Notch-6^{SPL}; M,N), PS-1 (15 ng PS-1^{ATG1}; O,P) or DAPT-treated (25 μM; Q,R) hypomorphant embryos. Staining was performed at 28 hpf (few hours prior to secondary sprout formation) for* EphrinB2a, Vegfr3 and Tbx20, *and at 48 hpf for* Dab2*, when arterial and venous differentiation of the DA and PCV were completed. Overall, expression of arterial markers in the DA and of venous markers in the PCV was comparable in control and morphant embryos. Also, note that there is no ectopic expression of these markers. The DA is indicated by a red vertical bar, while the PCV is denoted by a blue vertical bar. DA, dorsal aorta; PCV, posterior cardinal vein. Scale bar, representative for all panels: 100 μm.*

1.2.2 Notch receptors: Notch-1b (and Notch-6) regulate formation of the thoracic duct

Silencing of the different Notch receptors known in zebrafish using the mopholino knockdown strategy showed that knockdown of Notch-1a or Notch-5, zebrafish orthologues of mammalian Notch-1 and Notch-3, failed to cause any lymphatic defects (not shown). However, incomplete silencing of Notch-1b and, to a lesser extent, of Notch-6, impaired TD formation (Figure 15). Of note, the mammalian orthologues of these genes, Notch-1 and Notch-2, respectively, are expressed in LECs [47,231,232]. Complete loss of TD formation was observed in 30% of the embryos at the highest dose of the Notch-1b^{SPL} morpholino (15 ng; Figure 15C). In another 31% of Notch-1b^{SPL} embryos, the TD formed in only one to three of the ten somites analyzed. Similar lymphatic defects were obtained with a morpholino directed against the transcription start site (Notch-1b^{ATG}) (not shown). The TD also failed to form in 14% of Notch-6^{SPL} embryos (15 ng; Figure 15C). Combined silencing of Notch-1b and Notch-6 yielded more severe lymphatic defects, suggesting cooperation of both receptors (not shown). Since Notch-1b down-regulation resulted in more penetrant defects in lymphatic development, we further focused our analysis on Notch-1b^{KD}. Incomplete

knockdown of the Notch receptors also did not affect, or only minimally influenced the formation, differentiation and morphogenesis of blood vessels (Figure 14I-N and not shown).

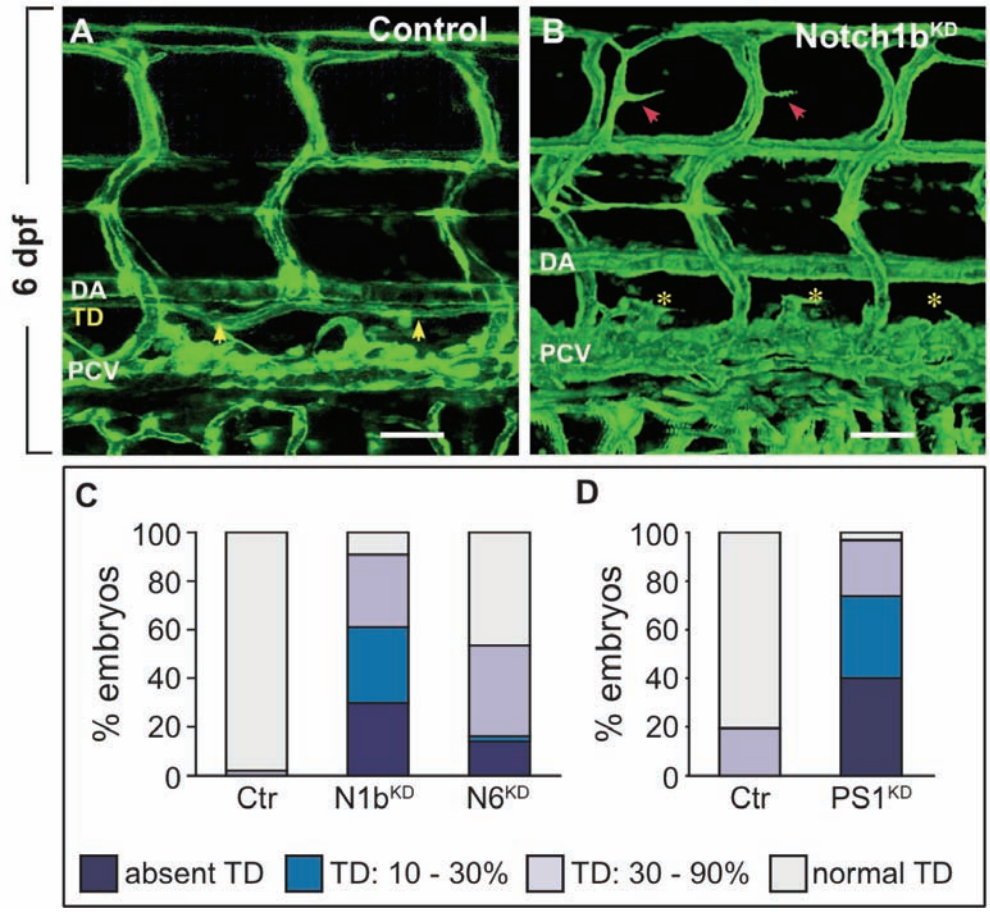

Figure 15: Inhibition of Notch-1b, Notch-6 or PS-1 impairs TD formation: A-B, *Confocal images of GFP^{+} vessels in the trunk of 6-dpf* Fli1:eGFP^{y1} *embryos. The control embryo has a normal TD (yellow arrowheads in A), while the TD is entirely absent in the Notch1b^{KD} (B). Yellow asterisks denote absence of the TD; red arrowheads indicated minimal hyperbranching of the upper part of the ISVs.* ***C,*** *Bar graphs, representing the percentage of embryos that develop a normal TD (grey), a TD over 30-90% (lilac) or over 10-30% (blue) of its normal length, or completely lack any TD (dark blue) in the indicated fraction of control embryos (*N=*185 in C, 78 in D) or Notch-1b^{KD} (N1b^{KD}) embryos (*N=*84; 15 ng Notch-1b^{SPL};* P<*0.001* versus *control; C), Notch-6^{KD} (N6^{KD}) embryos (*N=*63; 15 ng Notch-6^{SPL};* P<*0.001* versus *control; C), PS-1^{KD} embryos (*N=*65; 2.5 ng PS-1^{ATG1};* P<*0.001* versus *control; D). Scale bars: 50 μm*

1.2.3 Notch activators and mediators

Presenilin-1 (PS-1) is the catalytic subunit of the γ-secretase complex that activates Notch through proteolytic cleavage [278]. Consistent with the results obtained upon treatment with DAPT, silencing of PS-1 by injection of an ATG-blocking or a splice-binding morpholino similarly impaired TD formation (Figure 15D and not shown), without deregulation of blood vessel differentiation (Figure 14O-P). The role of PS-1 was specific, as silencing of PS-2 did not cause lymphatic defects, neither did combined double knockdown of PS-1 and PS-2 exacerbate the PS-1^{KD} phenotype (not shown). Therefore, PS-1 but not PS-2 seems to be involved in lymphatic development.

Suppressor of Hairless (Su(H)) is the transcription factor involved in canonical Notch signaling. In zebrafish, two isoforms of Su(H) are present, Su(H)a and Su(H)b. A morpholino targeting both isoforms was lethal at low concentrations. Silencing of the single Su(H) isoforms at concentrations that did not induce malformation in most of the injected embryos did not induce lymphatic defects (not shown). This could argue against a role for canonical Notch signaling in lymphatic development. However, we cannot exclude that higher dosages of morpholino can impair lymphangiogenesis. Su(H) binds to recognition sequences in the promoters of Notch target genes. When Notch signaling is not activated, the Su(H) transcription factor is bound by several co-repressors. Upon activation of the Notch receptor the intracellular domain binds the DNA-bound Su(H) transcription factors, thereby replacing the co-repressors and recruiting co-activators [112]. Thus, Su(H) functions both as an transcriptional repressor and activator during inactive and active Notch signaling, respectively. It is therefore not surprising that knockdown of Su(H) induces more severe defects than general inhibition of the Notch pathway by DAPT treatment. If Su(H)^{KD} yields too severe embryonic malformations, this precludes trustworthy analysis of the lymphatic vasculature.

2 NOTCH SIGNALING REGULATES EARLY STAGES OF LYMPHATIC DEVELOPMENT IN ZEBRAFISH

2.1 NOTCH SIGNALING IS CRITICAL DURING EARLY STAGES OF LYMPHATIC DEVELOPMENT

Having established that Notch signaling is a conserved mechanism in aquatic species, required for the formation of lymph vessels, we sought to explore the mechanisms whereby Notch regulates lymphangiogenesis. We first analyzed in zebrafish whether Notch signaling is required during the early steps of lymphatic development or rather regulates migration and assembly of LECs in a continuous TD (which starts at 3 dpf and is nearly accomplished in the trunk by 4 dpf). We therefore exposed zebrafish embryos to 25 µM DAPT at various developmental stages between 24 and 72 hpf. As mentioned above, the early treatment stages frequently induced other developmental defects leading to malformations, loss of blood circulation and edema. However, only the least affected embryos with a largely normal morphology and blood circulation in the major axial vessels at 4 dpf were analyzed for TD formation. This experiment indicated that DAPT inhibited the formation of the TD maximally when added during the initial processes of lymphatic development (24-30 hpf), but became progressively less effective at later stages (Figure 16). DAPT treatment at 56 hpf still resulted in 20% of embryos (N = 50) with complete abortion of TD formation (Figure 16). At this stage PL string formation is nearly completed in control embryos and LISV-PL migration is being initiated. These findings indicate that Notch signaling is important during the earliest stages of lymphatic development, but also seems to play a role beyond PL string formation. Remarkably, DAPT treatment at 72 hpf was ineffective in blocking outgrowth and assembly of the first TD fragments into a continuous TD (Figure 16). This was not due to insufficient inhibition of Notch signaling at 72 hpf, as zHer1 expression in embryos treated with 25µM DAPT at 72 hpf was still significantly decreased to 26% of the expression level in control embryos (N=3; $p<0.05$) as indicated by RT-PCR analysis at 4 dpf (not shown).

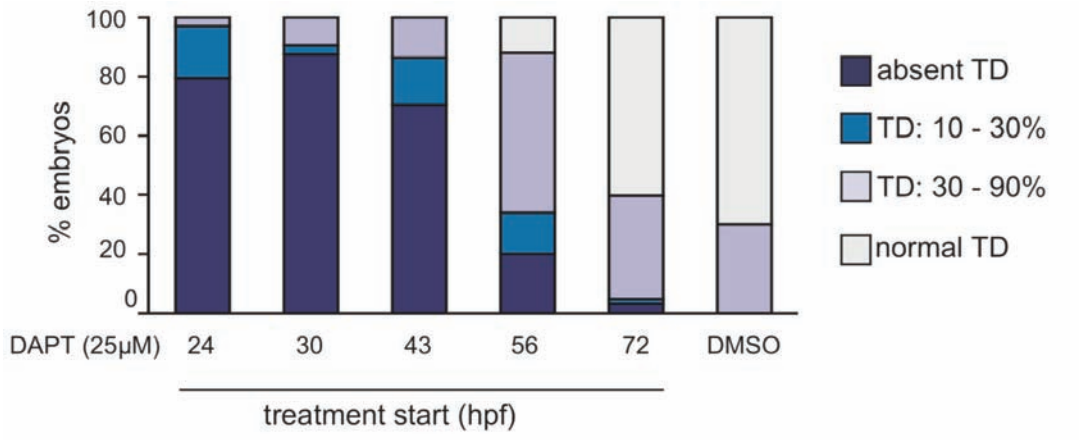

Figure 16: the effect of Notch inhibition by DAPT on lymphatic development becomes less pronounced at later stages. *Bar graphs, representing the percentage of embryos that develop a normal TD (grey), a TD over 30-90% (lilac) or over 10-30% (blue) of its normal length, or completely lack any TD (dark blue) in the indicated fraction of embryos treated with DMSO (N=30) or 25µM DAPT started at 24 hpf (N=30), 30 hpf (N=32), 43 hpf (N=44), 56 hpf (N=50) or 72 hpf (N=66). Treatments started at 24, 30, 43 and 56 hpf resulted in significantly different distributions of TD formation compared to DMSO treated controls (P<0.001).*

2.2 DLL4/NOTCH SIGNALING IS REQUIRED FOR PL STRING FORMATION

Since DAPT treatment results in overall blockade of Notch signaling and also inhibits other γ-secretase dependent pathways, this pharmacological approach likely also affects other developmental processes. Therefore, we further proceeded by analyzing the effect of morpholino knockdown on the different steps of lymphatic development.

As described above LECs from the TD are derived from the PL string, a transient source of LEC precursors at the horizontal myoseptum (Figure 3). To characterize the role of Notch signaling in earlier steps of lymphatic development, we first analyzed whether silencing of Notch also impairs development of PL cells, as they directly contribute to TD formation. Since PL cells develop initially from lymphangiogenic secondary sprouts in a segmented pattern, we used a similar quantification method as employed for analysis of the TD. At 52 hpf, formation of the string of PL cells was still proceeding in control embryos

(already completely formed in 53% and largely completed in another 40% of the embryos; Figure 17A-B). By contrast, in Dll4^{SPL} embryos, the PL string was completely absent in 38% and formed only in a few segments in another 27% of embryos (Figure 17A,C). Notably, largely comparable fractions of Dll4^{SPL} embryos exhibited similar types of severe, intermediate or subtle PL string and TD lymphatic defects (compare Figure 17A with Figure 13C), suggesting that the TD defects were, at least in part, attributable to defects in the initial formation of the PL string.

A similar absence of the PL string was observed when using the Dll4^{ATG} morpholino (not shown), or upon knockdown of Notch-1b (Figure 17A) or Notch-6 (not shown). These findings indicated that Notch signaling acts in part at very early steps of lymphatic development prior to formation of the perfused lymphatics.

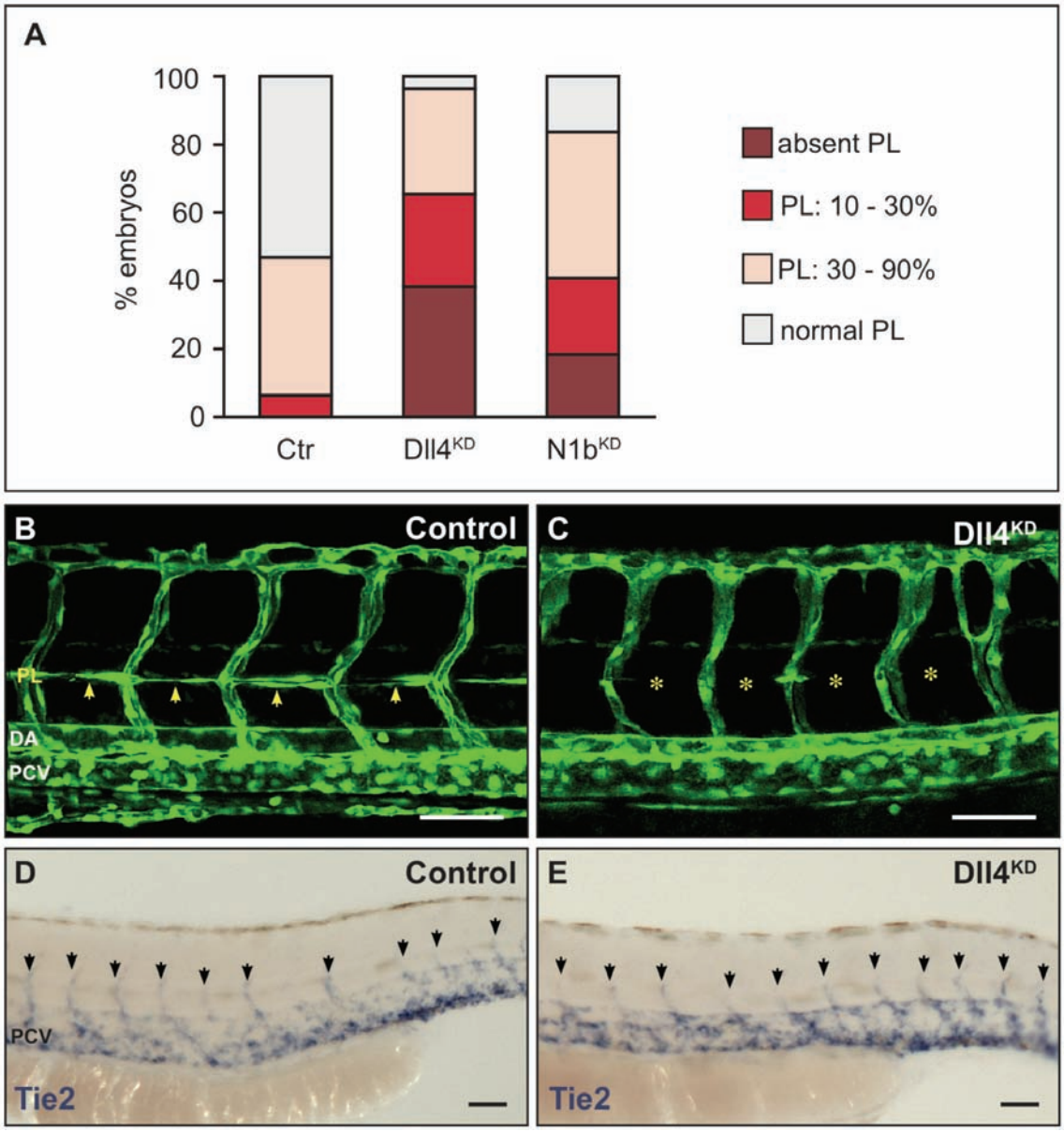

Figure 17: Incomplete silencing of Notch blocks PL string formation A, *Bar graphs, representing the percentage of embryos at 52 hpf, which develop a normal PL (grey), a PL over 30-90% (pink) or over 10-30% (red) of its normal length, or completely lack any PL (dark red) in the indicated fraction of control embryos (*N=*73), Dll4^{KD} embryos (*N=*55; 10 ng Dll4^{SPL};* P<*0.001* versus *control) or Notch-1b^{KD} (N1b^{KD}) embryos (*N=*49; 20 ng Notch-1b^{ATG};* P<*0.001* versus *control). **B,C,** Confocal images of Fli1:eGFP^{y1} embryos at 56 hpf, showing normal PL formation in the control embryo (arrowheads in B), but complete absence in the Dll4^{KD} embryo (asterisks in C). **D,E,** Whole-mount* in situ *staining for Tie2 at 50 hpf, revealing a normal number of secondary sprouts (arrowheads) in the control (D) and Dll4^{KD} (E) embryo. Scale bars: 50 μm*

2.3 DLL4 SILENCING REDUCES THE FRACTION OF LYMPHANGIOGENIC SECONDARY SPROUTS

Since the PL string at the horizontal myoseptum is formed by lymphangiogenic secondary sprouts from the PCV, we also studied whether inhibition of Notch signaling might even act at an earlier stage, i.e., during branching of these lymphangiogenic secondary sprouts. Whole-mount staining for *Tie2* [#], which marks all secondary sprouts [92], showed a normal total number of secondary sprouts from the PCV in all Dll4^{KD} embryos analyzed (*N*=20; 50 hpf; Figure 17D-E). Half of these secondary sprouts, i.e. the angiogenic sprouts, normally connect to the primary arterial ISVs that loose their arterial connection to the DA and become venous ISVs (Figure 3). The other half of the secondary sprouts, the lymphangiogenic sprouts grow to the level of the horizontal myoseptum where they deliver the PL cells after which the connection between the PL string and the PCV disappears (Figure 3). Despite the normal total number of secondary sprouts, high resolution imaging of the trunk vasculature in 4-dpf *Fli1:eGFP^{y1}* embryos revealed alterations in the proportion of primary ISVs that adopted a venous phenotype (vISVs), based on the criterion that they were connected to the PCV. As expected, in control embryos, approximately half of the ISVs were connected to the PCV (vISVs, % of total ISVs: 54 ± 1.3%; *N*=49 embryos). In contrast, in Dll4^{SPL} embryos, a larger fraction of ISVs was connected to the PCV and therefore venous (vISVs, % of total ISVs in all Dll4^{SPL} embryos analyzed: 82 ± 1.8%; *N*=97, *P*<0.05 versus control). Similar findings were obtained in Notch-1b^{SPL} embryos (vISVs, % of total: 69 ± 2.7%; *N*=27, *P*<0.05 versus control). These findings, and the observation that silencing of Dll4, Notch-1b or Notch-6 aborted PL string formation in a substantial fraction of morphant embryos, indicated that a fraction of secondary sprouts, that would normally have been lymphangiogenic, were angiogenic, thereby impairing TD formation (see scheme in Figure 25A,B).

We provided additional experimental support for the aforementioned model by using high resolution video-imaging of a double transgenic reporter line, the *Flt1:YFP*x*kdr-l:mCherryRed* line [#]. The *kdr-l:mCherryRed* transgene labels arterial and venous ECs, whereas the *Flt1:YFP* marker exclusively labels arterial ECs [90]. Hence, in the *Flt1:YFP*x*kdr-*

[#] whole-mount *in situ* staining for Tie2 and imaging of the Flt1:YFPxkdr-l:mCherryRed line was performed by the Schultle-Merker lab (Hubrecht Institute-KNAW and University Medical Center, Utrecht, the Netherlands)

l:mCherryRed line, venous cells are red (CherryRed^{+}) and arterial cells are yellow (YFP^{+}CherryRed^{+}) in merged images. Once a primary aISV adopts a venous identity when being “co-opted” by a secondary venous angiogenic sprout, this vessel gradually changes its pattern of marker expression and becomes venous. In control embryos, those primary aISVs that became connected with a venous secondary sprout and thus adopted a venous identity, indeed progressively lost their “arterial” yellow color and acquired a “venous” red color (blue arrows in Figure 18A-A”), while the other half of the aISVs remained connected with the DA and retained their yellow color (white arrows in Figure 18A-A”). Closer inspection revealed that, upon establishing a connection with a venous secondary sprout, the arterial ECs of the aISV became gradually replaced by venous ECs from the secondary sprout in a ventral-to-dorsal direction. In contrast, in Dll4^{KD} embryos with severe lymphatic defects, nearly all yellow aISV connections with the DA disappeared (see the single white arrow in Figure 18B-B”), and were gradually replaced by red vISV connections with the PCV (blue arrows in FigureB,B”). Thus, the imagings confirmed that a supernumerary fraction of angiogenic sprouts is formed in Dll4^{KD} embryos at the expense of lymphangiogenic sprouts, that would otherwise go on to form the PL string.

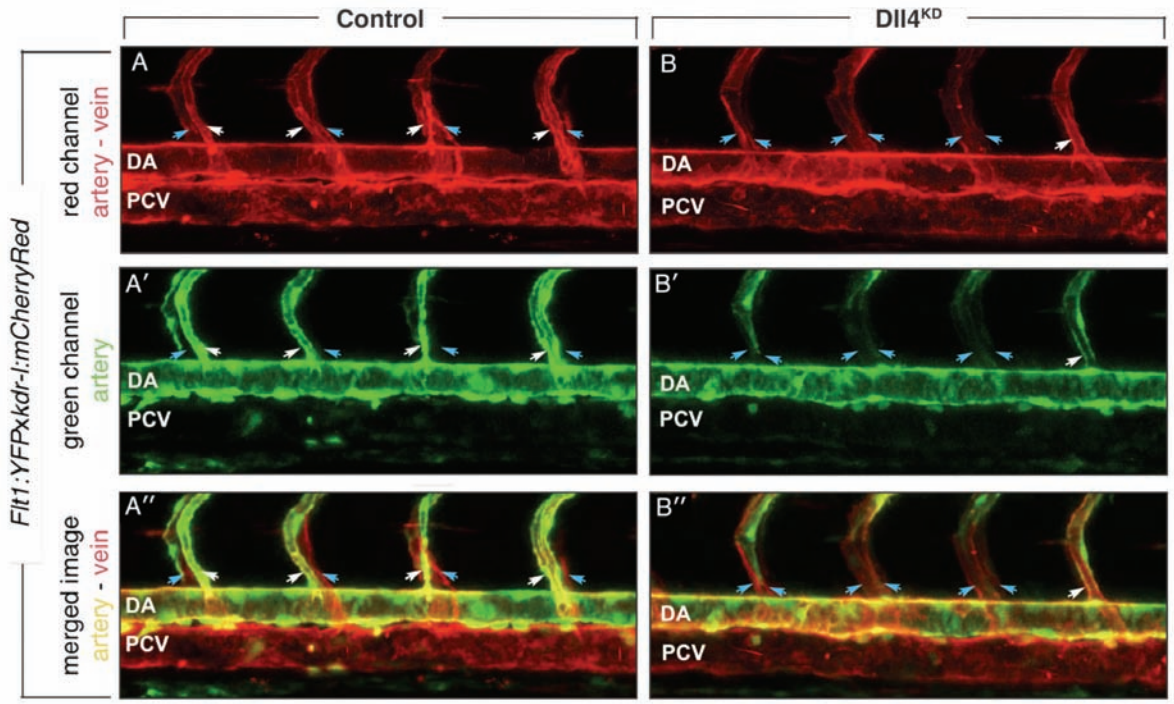

Figure 18: Notch silencing reduces the fraction of lymphangiogenic sprouts. ***A,B*** *Confocal images of the trunk vasculature in* Flt1:YFPxkdr-l:mCherryRed *embryos, where* kdr-l:mCherryRed *marks both venous and arterial vessels in red (A,B),* Flt1:YFP *labels arterial vessels in green (A',B'), and the merged images show arterial vessels in yellow (green-red) and venous vessels in red (A",B"). Lateral view images of the entire embryo were taken, such that the ISVs at both the left and right side of the embryo are shown, partially superimposed onto each other. Embryos were imaged at 54 hpf, when secondary sprouts had already connected to the primary ISVs, and the latter were in the process of changing their arterial to venous identity in a ventral-to-dorsal pattern. In control embryos (A-A"), on average half of the aISVs became connected to the PCV and thus acquired a venous identity, thereby progressively losing their green arterial signal (blue arrows in A') and thus becoming marked in red only (blue arrows in A,A"), while the other half of the aISVs remained connected to the DA and labeled in green (white arrows in A') and thus in yellow on the merged images (white arrows in A"). By contrast, in the Dll4^{KD} embryo shown (B-B"), most ISVs lost their green arterial marker in a ventral-to-dorsal direction (blue arrows in B',B"; note the single white arrow), and became marked in red only, while loosing their yellow label (blue arrows in B"). White arrow in B-B" denotes a residual aISV retaining its green (B') or yellow (B") label. Scale bar in A, representative for all panels, is 50 μm.*

2.4 ACTIVATION OF NOTCH BY DLL4 PROMOTES LYMPHATIC CHARACTERISTICS *IN VITRO*

To evaluate whether activation of Notch in venous ECs would induce lymphatic properties, we cultured human umbilical venous ECs (HUVECs, which express Notch receptors including *NOTCH-1*, but negligible levels of *PROX-1*; not shown) on a feeder layer of monkey COS cells, expressing DLL4 (COS^{Dll4}) or a control vector (COS^{CTR}), and analyzed by RT-PCR with human gene-specific primers the expression of lymphatic markers. As shown in Figure 19, several well-known direct Notch target genes (*HEY1*, *HEY2*, *HES1*, *NRARP*) were upregulated in HUVECs, when cultured on COS^{Dll4} feeder cells as compared to control COS^{CTR} feeder cells, indicating that Notch signaling was activated. Furthermore, COS^{Dll4}-activated HUVECs expressed elevated levels of the lymphatic markers *PROX-1*, *VEGFR3*, *LYVE-1* and *SOX18* (Figure 19). Expression of *EPHRINB2* has been implicated in development of both arterial vessels and collecting lymphatics (not capillaries) [70,172]. Furthermore, it should be noted that *EPHRINB2* has also been shown to be a direct Notch target gene in endocardial cells, having CSL-binding sites in its promoter [279] and could therefore also be considered to belong to the group of direct Notch targets. Expression of *COUP-TFII,* which is expressed in both venous and lymphatic ECs [234], is also upregulated, but levels of other blood vessel markers (*NEUROPILIN-1*, *ENDOGLIN, VE-CADHERIN*, *CD31*) were not or only minimally affected (Figure 19). This upregulation of lymphatic markers was abolished by treatment of the cells with DAPT (30 µM; not shown).

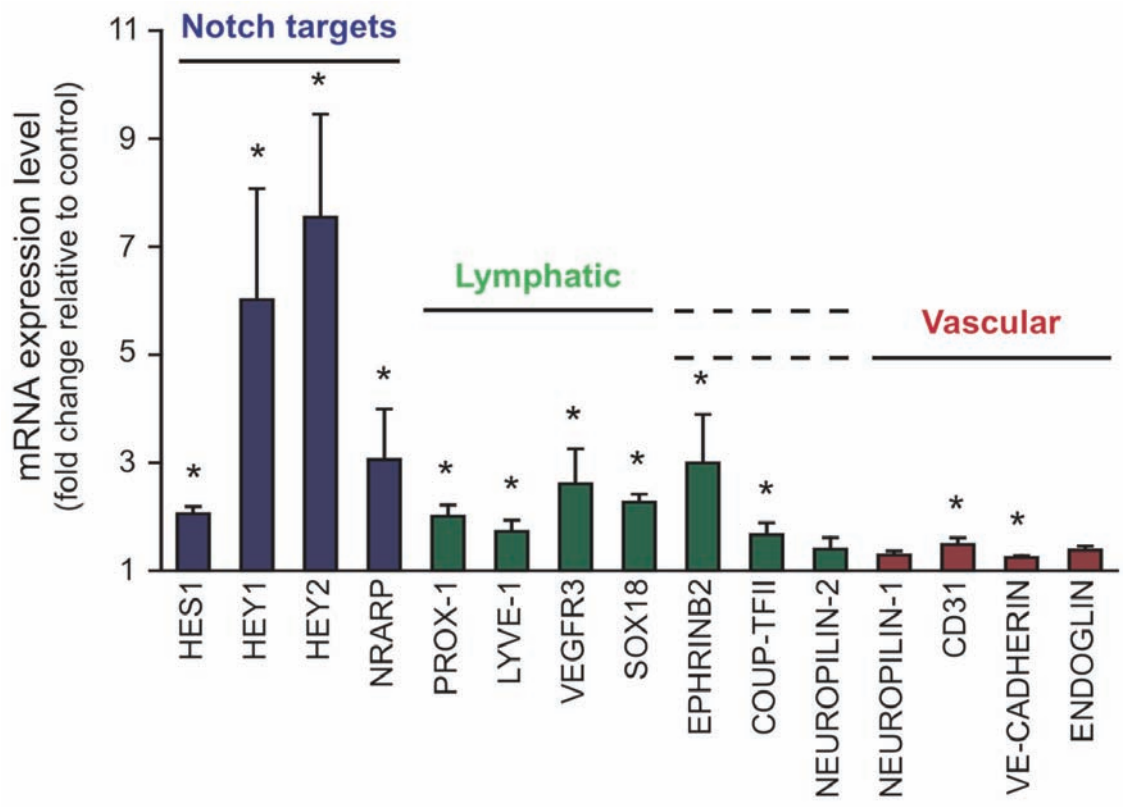

***Figure 19: Activation of Notch by Dll4 promotes lymphatic characteristics* in vitro.** *Quantitative RT-PCR analysis of HUVECs, co-cultured with COS cells stably expressing hDll4 (COS^{Dll4}) or a control vector (GFP; COS^{CTR}), confirming upregulation of Notch target genes (*HES1, HEY1, HEY2, NRARP*; blue bars) and revealing enhanced lymphatic marker expression (*VEGFR3, PROX-1, LYVE-1, EPHRINB2, SOX18*; green bars), while vascular genes such as* NEUROPILIN-1, CD31, VE-CADHERIN *or* ENDOGLIN *(red bars) were only minimally affected.* COUP-TFII*, which is expressed in venous and lymphatic ECs, was also upregulated. Results are represented as fold change in HUVEC/COS^{Dll4} co-culture versus HUVEC/COS^{CTR}. Mean±SEM; N= 3-11; *, $p < 0.05$.*

2.5 IS NOTCH INVOLVED IN LYMPHATIC SPECIFICATION IN ZEBRAFISH?

The lymphatic differentiation process in mammals has been described to occur in different steps (reviewed in [39]; Figure 2). *Sox18* expression marks a subpopulation of venous ECs in the cardinal vein that are competent to obtain a lymphatic identity. An unknown factor or stimulus induces *Prox1* expression in a subset of these competent venous ECs, which initiates commitment towards LEC differentiation. These cells start migrating away from the

cardinal vein towards VEGF-C signals to form the initial lymph sacs. During migration these cells become even further committed, i.e. determined to the LEC lineage by expressing additional LEC markers, such as *Nrp2*, *Foxc2*, *Podoplanin*, *Ang2* and *EphrinB2*. From the lymph sacs, LECs sprout and migrate away to form the whole lymphatic vascular network. These growing lymphatic vasculature branches further mature and their LECs are only completely differentiated near the time of birth when they express the complete profile of lymphatic marker genes. Of all these lymphatically expressed genes, only overexpression of either Sox18 or Prox1 in BEC are shown to be sufficient to induce lymphatic differentiation [46,47,50]. Thus, Sox18 and Prox1 are, at present, the most important known regulators of lymphatic development. Therefore, in order to answer the question whether Notch is involved in the processes of lymphatic specification or commitment, we have analyzed the expression of *Sox18* and *Prox1* in control and Notch morphant embryos.

2.5.1 Sox18 expression

Sox18 has been described to be expressed in the DA and PCV at 24 hpf and in the primary ISVs sprouting from the DA [253,262,280]. We have obtained similar expression patterns by whole mount *in situ* staining for Sox18 at 30 hpf (Figure 20A). At 38 hpf *Sox18* expression is strongest at the interface between DA and PCV (Figure 20C). Both at 30 hpf and 38 hpf, stages spanning the secondary sprout formation from the PCV, we found no obvious alterations in the Sox18 expression pattern in control and $Dll4^{KD}$ embryos (Figure 20A-D), in agreement with previous findings that *Sox18* expression was not affected in *Mindbomb* mutants that lack an E3 ubiquitin ligase crucial for Notch signal activation [262].

However, it should be noted that a crucial role for Sox18 in zebrafish lymphangiogenesis, similar as in mice, has not been reported. To test this we have performed morpholino knockdown experiments using the previously described Sox18 morpholino that combined with Sox7 morpholino blocks arterial-venous differentiation in zebrafish [253,262]. Preliminary data using morpholino concentrations avoiding major vascular defects (15 ng $Sox18^{ATG}$) did not show lymphatic defects in PL string formation nor TD formation (not shown). However, as the zebrafish genome underwent a duplication, it is possible that an as yet unidentified paralogue, mediates lymphatic specification.

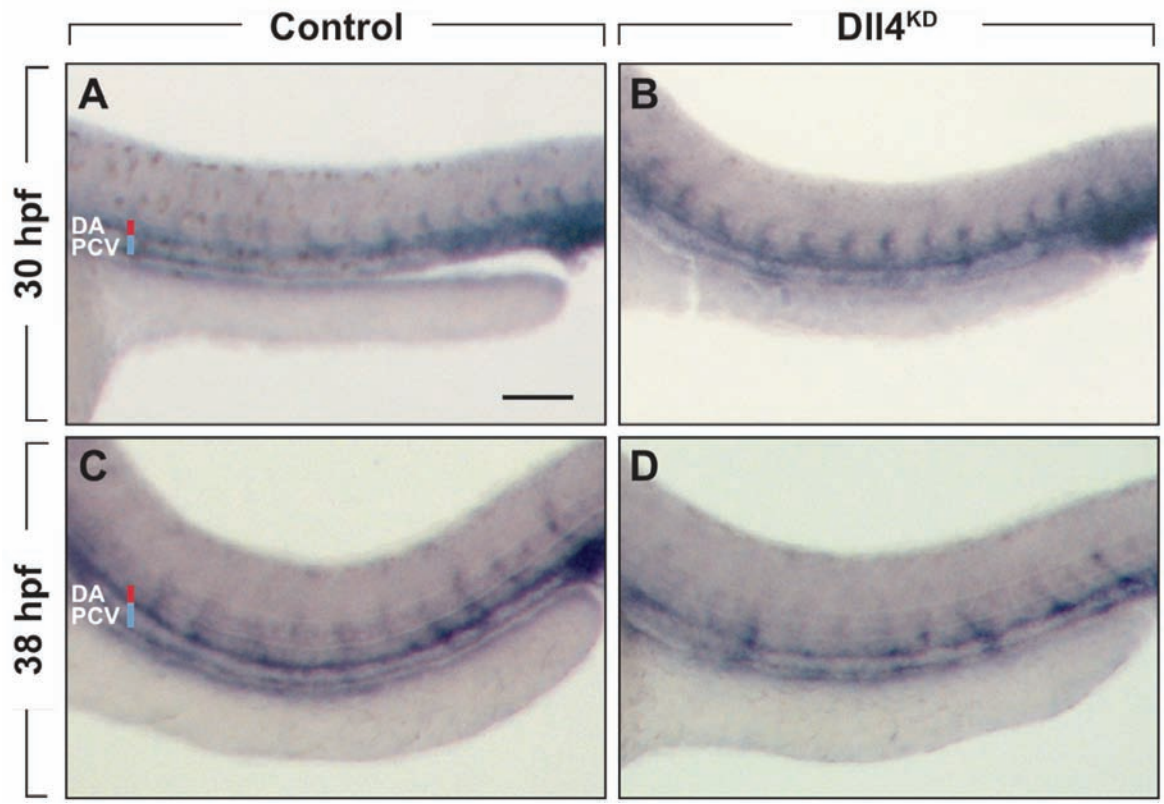

Figure 20: Sox18 expression in control and Dll4^{KD} embryos. A-D *Whole-mount* in situ *staining for Sox18 at 30 hpf (A,B) and 38 hpf (C,D) in control embryos (A,C) and Dll4^{KD} (10ng Dll4^{SPL}) embryos (B,D), revealing normal expression of Sox18 at stages of lymphangiogenic sprout formation. Red bar indicates dorsal aorta (DA), blue bar posterior cardinal vein (PCV). Scale bar: 100 μm.*

2.5.2 Prox1 expression

Previously, *Prox1* expression was shown in the PCV, secondary sprouts from the PCV, the PL string (then termed parachordal vessel) and thoracic duct [7]. However, despite numerous efforts and different staining techniques (whole mount ISH, ISH on cryosections, whole mount immunostaining, immunostaining on cryo- and paraffin sections) we [❖] and others (Schulte-Merker lab [#], Gerhardt lab [*], Cotelli lab [‡]) have not been able to reproduce

❖ we performed whole mount ISH, ISH on sections, immunostainings with different available antibodies

Schulte-Merker lab (Hubrecht Institute-KNAW and University Medical Center, Utrecht, the Netherlands) performed whole-mount *in situ* stainings for Prox1

* Gerhardt lab (London Research Institute–Cancer Research, London, England) performed whole mount immunostainings for Prox1

these described staining patterns in zebrafish. This was not due to failure of the staining procedures, as in all approaches (ISH, immuonstaining), the other reported sites of *Prox1* expression (e.g. eye, neural tube, lateral line, muscle pioneer cells, pronephros [266,281,282]) were stained (not shown). We have also analyzed the expression patterns of *Prox1-like* and *Prox2*, a paralogue and homologue, respectively, of *Prox1,* by whole-mount *in situ* staining to explore whether one of these zebrafish *Prox* genes might be the counterpart of mammalian Prox1 for zebrafish lymphangiogenesis. However, we did not detect expression in the lymphangiogenic and lymphatic structures at stages between 24 and 75 hpf (not shown). Several reasons can explain why we did not detect expression of any of the Prox1 homologous genes in zebrafish. It is possible that expression of Prox1 was below the threshold of detection or in case of the lymhangiogenic sprouts and PL string, structures were too small to detect labeled cells. It could also be that the currently known zebrafish Prox1 homologues are not involved in lymphatic development.

In an alternative strategy, we have tried to isolate the vascular and lymphatic EC fractions of *Fli1:eGFP*^{y1} embryos in order to perform RT-PCR expression analysis. Therefore, we adoped a technique that was established in our lab in the *Xenopus laevis* model ([257], manuscript in preparation) which makes it possible to specifically label LECs. Briefly, this LEC labeling is performed by injecting TRITC-dextran dye intramuscularly in *Fli1:eGFP*^{y1} zebrafish embryos. The dye is drained by the lymphatics and specifically taken up by LECs (but not BECs), via a process of pinocytosis ('cell drinking') (Figure 21). In this way LECs become labeled red by the presence of intracellular dye-containing vesicles. FACS sorting of the cells of these embryos enables the isolation of the double red/green LEC fraction and the single green BEC fraction. Unfortunately, this LEC labeling technique only works efficiently in 3 to 4 week old embryos, making it impossible to use this technique to isolate the early-stage lymphangiogenic or lymphatic ECs, e.g. from the PL string, LISV or early TD. However, RT-PCR analysis on the LEC fraction of older embryos confirmed the presence of *Prox1* mRNA in LECs in zebrafish, suggesting that, similar as in mammals, Prox1 is also a marker of lymphatic structures in zebrafish (not shown).

[‡] Cotelli lab (the Department of Biology, University of Milan, Milan, Italy) performed whole-mount *in situ* stainings for Prox1

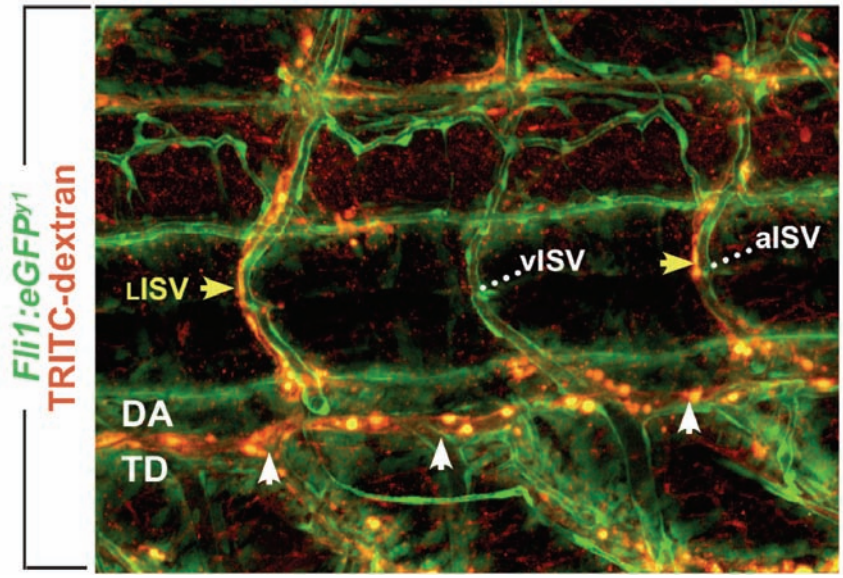

Figure 21: LEC labeling technique in zebrafish embryos. *A 4 week old Fli1:eGFP^{y1} zebrafish embryo was injected intramuscularly in the tail with TRITC-dextran. The dye is drained by lymphatics and taken up intracellularly by pinocytosis in LECs. In this way lymphatic structures (TD (thoracic duct), LISV (lymphatic intersomitic vessels) become labeled red making it possibly to separate the BEC fraction (GFP$^+$) from the LEC fraction (GFP$^+$/TRITC$^+$) by FACS sorting.*

2.5.3 Inhibition of Prox1 impairs thoracic duct development

The lack of an appropriate technique to detect *Prox1* expression in the early lymphangiogenic structures raised concerns about the role of Prox1 in lymphatic commitment in zebrafish embryos. We therefore analyzed the effect of Prox1 knockdown on lymphatic development. Injection of a transcription-blocking morpholino, Prox1^{ATG1}, resulted in dose-dependent impairment of both PL string and TD development. At the highest morpholino dose (10ng), TD formation was completely blocked in up to 45% of morphant embryos (*N*=86; Figure 22). Unfortunately, we were unable to confirm the specificity of these lymphatic defects with a second morpholino. A splice-blocking morpholino (Prox1^{SPL}) was too toxic and lethal at low dosages. A second, independent ATG-binding morpholino (Prox1^{ATG2}) did not impair TD formation. Therefore, we cannot draw firm conclusions on the role of Prox1 in lymphatic commitment in zebrafish.

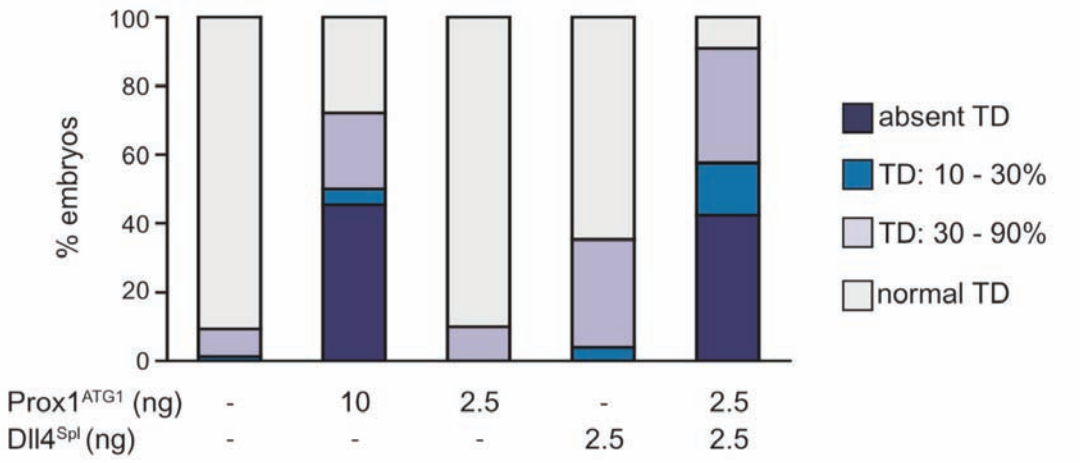

Figure 22: Dll4 and Prox1 synergistically regulate TD formation. *Bar graphs, representing the percentage of embryos that develop a normal TD (grey), a TD over 30-90% (lilac) or over 10-30% (blue) of its normal length, or completely lack any TD (dark blue) in the indicated fraction of control embryos (*N=*47) or embryos injected with a high dose of Prox1^{ATG1} (10ng, N=86), a low dose of of Prox1^{ATG1} (2.5ng; N=30), a low dose of Dll4^{SPL} (2.5ng; N=51) or a combination of these low dosages of Prox1^{ATG1} and Dll4^{SPL} (N=33). TD defects after injection of a high dosage of Prox1^{ATG1}, a low dosage of Dll4^{SPL} or combined injection of Prox1^{ATG1} and Dll4^{SPL} were significantly different from control injections (*P*<0.001).*

2.5.4 Synergistic impairment of lymphatic development upon combined knockdown of Prox1 and Dll4

Notwithstanding this controversy, we made use of the Prox1^{ATG1} morpholino to study whether Prox1 knockdown would aggravate Dll4 morphant phenotypes. Therefore, the effect of combined knockdown of Prox1 and Dll4 was analyzed using morpholino doses that did not or only minimally impair lymphatic development in the single knockdowns (2.5ng Dll4^{SPL}; 2.5 ng Prox1^{ATG1}; Figure 22). Whereas single knockdown of either Dll4 or Prox1 did not or only minimally impair TD development at these low morpholino dosages (*N*= 51 and 30, respectively; Figure 22), combined knockdown completely blocked TD formation in 42% of embryos and reduced TD formation to less than 30% of its normal length in another 15% of embryos (*N*=33) (Figure 22). Similarly, PL formation was not impaired in the single knockdowns, but was completely blocked in 23% of double knockdowns and reduced to less

than 30% of its normal length in 31% of embryos (*N*=39) (not shown). These findings suggest that Prox1 and Dll4 act synergistically in regulating lymphatic development in zebrafish embryos.

Together, the findings that Dll4-mediated activation of Notch signaling in BECs *in vitro* induces upregulation of lymphatic marker genes and that Prox1 and Dll4 synergistically regulate lymphatic development in zebrafish embryos, suggest a potential role for Dll4/Notch signaling upstream of or in parallel with Prox1 in lymphatic commitment. This would also explain the reduced formation of lymphangiogenic secondary sprouts upon Dll4/Notch inhibition. However, these data should be interpreted with care in view of the absence of confirmation by a second Prox1 morpholino.

3 INHIBITION OF NOTCH SIGNALING IMPAIRS LYMPHATIC NAVIGATION

3.1 SILENCING OF DLL4 IMPAIRS PL CELL MIGRATION ALONG AISVS

We also explored whether Notch signaling might regulate the development of the TD by controlling LISV formation or migration, as the TD failed to form in a substantial fraction of Dll4^{SPL} embryos (25%), even despite the fact that its precursor, the PL string, developed partially in these embryos. Also, as described above, addition of DAPT to zebrafish embryos at 56hpf, when the PL string is nearly completely formed, still completely blocked PL formation in 20% of treated embryos (Figure 16). High-resolution imaging revealed several types of LISV abnormalities in Dll4^{KD} embryos, the most frequent being absence of LISVs along ISVs. Intriguingly, we have observed that in control embryos, LISV-PLs only migrated along aISVs but never along vISVs, suggesting that vISVs are not permissive (Figure 23A-B). Since there were more vISVs and fewer aISVs in Dll4^{KD} embryos, migrating LISV-PLs were deprived from their usual arterial template and could therefore not contribute to the formation of the TD (Figure 23C). Intriguingly, however, we also noticed that, even when residual aISVs formed in Dll4^{SPL} embryos, LISV-PLs bypassed the aISV post, and failed to

turn and migrate along aISVs (Figure 23D). Indeed, when analyzing the fraction of aISVs that was accompanied by LISV-PLs in Dll4^{SPL} embryos with a nearly complete PL string (over >90% of its length), as much as 49 ± 6% of these aISVs were not accompanied by LISV-PLs in Dll4^{SPL} embryos (*N*=205 aISV), while only a few aISVs lacked accompanying LISV-PLs in control embryos (15 ± 4%; *N*=334 aISV; *P*<0.05).

Other, much less frequent LISV defects included LISV-PLs that made the turn alongside the aISV, but stalled (Figure 23E), or even, in a few cases, misrouted LISV-PLs migrating along vISVs (Figure 23F). Stalling was observed for 9.2% and 2.7% of LISV that migrated up- or downward, respectively, along aISV in Dll4^{KD} embryos (*N*=293 aISV), as compared to 1.8% and 0.7%, respectively, in control embryos (*N*=450 aISV). Misrouting along vISVs was only observed in 0.25% of vISVs in Dll4^{KD} embryos (*N*=1617 vISV), however, this rare aberrant phenotype was never observed in control embryos (*N*=767 vISV).

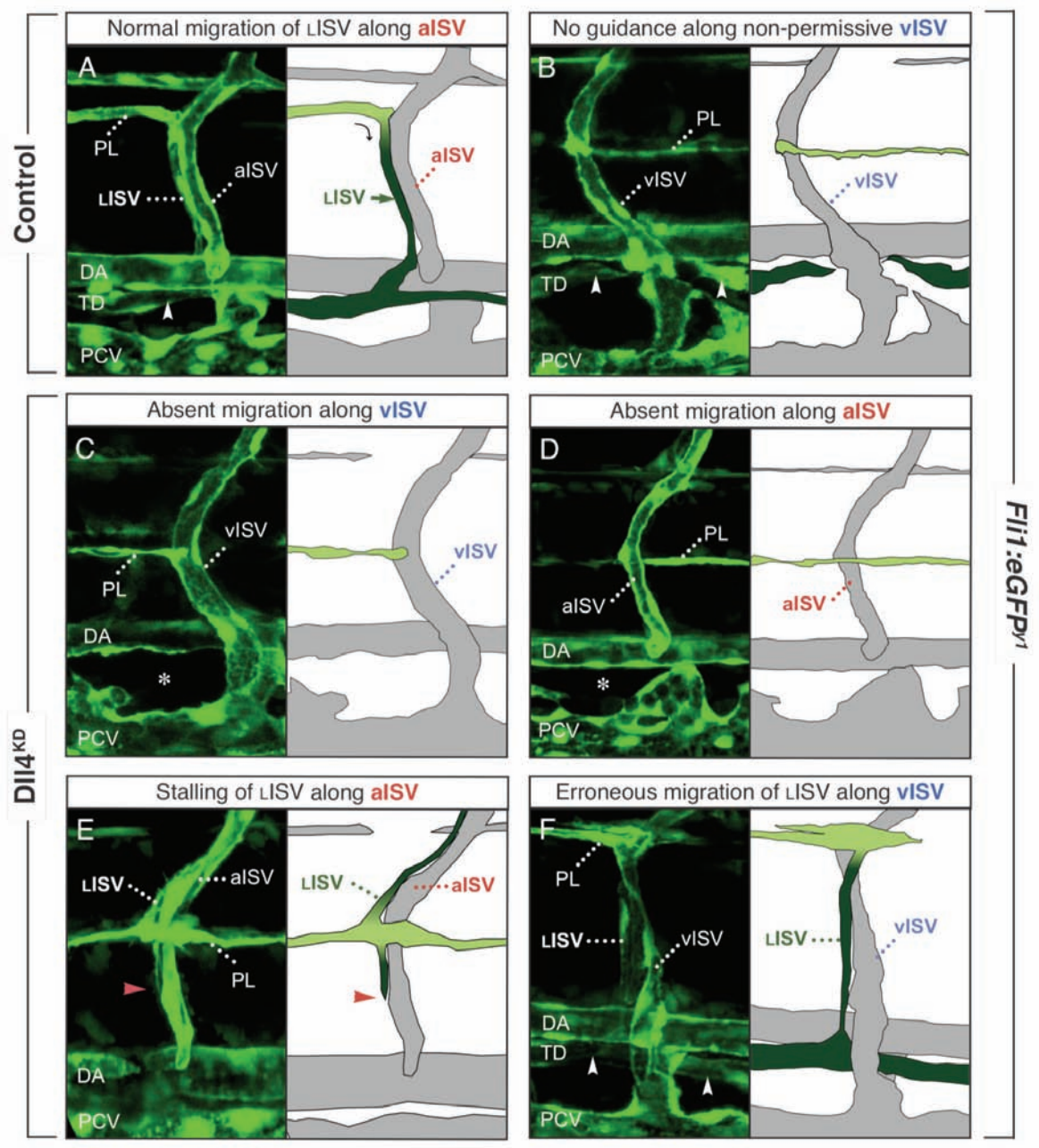
Control
Dll4KD
Fli1:eGFPy1
Normal migration of LISV along aISV
No guidance along non-permissive vISV
Absent migration along vISV
Absent migration along aISV
Stalling of LISV along aISV
Erroneous migration of LISV along vISV
A
B
C
D
E
F
PL
LISV
aISV
vISV
DA
TD
PCV

Figure 23: Incomplete silencing of Notch perturbs lymphatic navigation. *Panels A-F are confocal images with accompanying schematic redrawing of the navigation routes of LISVs along aISVs or vISVs in control (A,B) and Dll4^{KD} (C-F)* Fli1:eGFP^{y1} *embryos at 4 dpf. Permanent lymphatic structures (LISV, TD) are labeled dark green; transient lymphangiogenic structures (PL) are labeled light green.* ***A,B,*** *In control embryos, LISV-PLs navigate alongside aISVs and establish a continuous TD (arrowhead). Note how the LISV "creeps" over its aISV guidance template (A). LISV-PLs never navigate along vISVs in control embryos (B).* ***C-F,*** *Different types of navigation defects in Dll4^{KD} embryos.* ***C,*** *In a large fraction of morphant somites, LISV-PLs lacked migration templates because fewer aISVs developed. As LISV-PLs do not normally migrate along vISVs, no TD was formed in these somites (asterisks).* ***D,*** *In other morphant somites, LISV-PLs bypassed the point of turning at the aISVs, and failed to switch to radial migration.* ***E,*** *In a small fraction of somites in Dll4^{KD} embryos, LISV-PLs accomplished to make the turn and switched to radial migration, but then stalled shortly thereafter (red arrowhead denotes the arrested tip of a navigating LISV).* ***F,*** *In most Dll4^{KD} embryos, vISVs were not permissive to guide LISV-PLs, but, occasionally, LISV-PLs erroneously navigated alongside a vISV.*

3.2 NOTCH INHIBITION DOES NOT AFFECT INTRINSIC MOTILITY PROPERTIES OF LECS *IN VITRO*

In the above-described defective migration phenotypes of absent or stalled LISV-PLs along residual aISVs templates (Figure 23C,E), LISV-PLs are present, however, they do not migrate. We have performed various *in vitro* studies to examine whether the intrinsic properties of LECs were altered after Notch inhibition and could therefore explain the defective migration of these cells.

We have analyzed the effect of Notch inhibition and activation on cellular migration and motility by using different experimental setups. In the first place, we have performed a scratch wound migration assay in which the rate is measured at which a gap in a confluent layer of LECs is closed. Inhibition of Notch signaling by treatment with DAPT had no effect on the rate of gap closure (Figure 24A). In agreement, neither activation of Notch signaling by growing the cells on Dll4-Fc coated plates [206] nor inhibition of Notch signaling by treatment with DAPT showed an effect in a transwell migration experiment that measured the migration of LECs through a porous membrane (Figure 24B-C).

We also analyzed the effects of Notch inhibition on proliferation of LECs to verify (in an *in vitro* set up) whether reduced proliferation properties of PL cells upon Notch inhibition could explain the reduced presence of migrating LISV-PLs. Proliferation was measured by quantifying the amount of viable cells after culturing LECs for 48 hours in the presence or absence of DAPT. No effect of Notch inhibition on proliferation of LECs was found (Figure 24D).

Finally, we determined the effect of Notch inhibition on sprouting and capillary tube formation. LECs were coated on beads and embedded in a collagen gel. Sprouting was induced by addition of full growth factor supplemented medium. Addition of DAPT did not affect the length of the sprouts but an increased number of sprouts was observed (not shown), reminiscent of the increased vascular sprouting phenotype upon Notch inhibition [103]. However, *in vivo*, we had not observed increased sprouting from or hypberbranching of the PL string in zebrafish upon Notch inhibition.

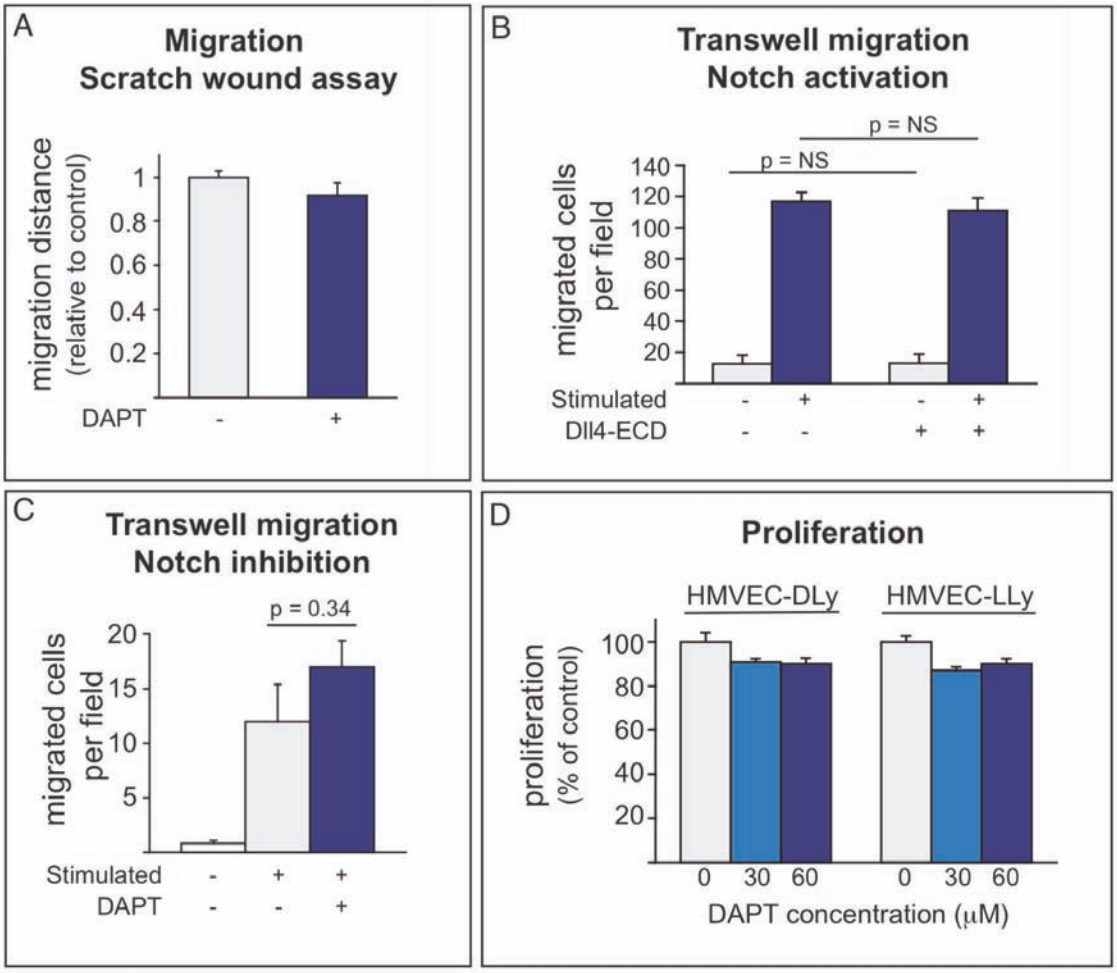

Figure 24: Inhibition of Notch does not affect in vitro proliferation and migration. A*, Migration of LECs, analyzed using a scratch wound healing assay, was not inhibited by DAPT (30 μM).* ***B****, Transwell migration of LECs in response to culture medium containing 10% FBS and 100 ng/ml VEGF-C in the lower compartment was comparable, when cells were seeded on filters coated with BSA (control) or the extracellular domain of Dll4 (Dll4-ECD), previously shown to activate Notch signaling.[9]* ***C****, Transwell migration of LECs in response to culture medium containing 10% FBS and 100 ng/ml VEGF-C in the lower compartment ("stimulated") was not affected by DAPT (60 μM).* ***D****, Primary LECs of dermal (HMVEC-DLy) and lung (HMVEC-LLy) origin were starved overnight, after which proliferation was induced with full growth medium with or without increasing concentrations of DAPT (30-60 μM). Proliferation was measured as the number of viable cells after further culturing for 48 hours, expressed in % of control. Error bars represent SEM;* N=*5-11.*

These *in vitro* evidences indicate that the intrinsic properties regulating movement of LECs were not affected. This may imply alterations in the migration scaffolds/templates formed by the aISV (see above). However, the arterial identity of the guiding ISVs does not seem to be affected. Indeed, despite their reduced number, the remaining aISV showed normal expression of the arterial marker *Flt1* in the *Flt1:eGFP* reporter line [#] (Figure 18B',B"). Furthermore, initial arterial/venous differentiation was normal as shown by the normal expression of arterial and venous marker genes in the differentiated DA and PCV at 28hpf at the highest morpholino and DAPT dosages used (Figure 14). Therefore, we believe that the reduction of migrating LISV-PLs is due to defective turning of LISV-PLs from tangential migration at the horizontal myoseptum to radial migration along the arterial guidance template and that this is probably due to loss of a recognition signal. This recognition signal might be Notch itself or an as yet unknown factor regulated by Notch signaling.

4 EXPRESSION OF DLL4 AND NOTCH-1B IN ZEBRAFISH

To examine the expression of Dll4 and Notch in order to relate it to the observed lymphangiogenic/lymphatic defects, we used whole-mount *in situ* hybridization in control embryos during the developmental processes that were affected in Notch hypomorphants. At 30 hpf, when secondary sprout formation starts, Dll4 was strongly expressed in the DA but undetectable in the PCV (Figure 25A,B), consistent with previous findings [92,189,190]. Notch-1b was strongly expressed in the DA (Figure 25C,D), while a much weaker signal appeared dispersed in certain endothelial cells of the dorsal part of the PCV, though the low Notch-1b signal approached the detection limit of available techniques (Figure 25E). Expression of Notch-6 was at or below the threshold of detection of the *in situ* hybridization technique; when expression was detectable, a weak signal was observed in the axial vasculature consistent with its previously reported expression pattern (not shown) [283]. Thus,

[#] Imaging was performed by the Schultle-Merker lab (Hubrecht Institute-KNAW and University Medical Center, Utrecht, the Netherlands)

ligand and receptor appear to be expressed in the near vicinity of the emerging lymphangiogenic secondary sprouts. The limited resolution of *in situ* hybridization (whole-mount or sections) did not allow us to evaluate possible expression in the thin lymphangiogenic secondary sprouts or the PL string.

Dll4 and Notch-1b were also detected in primary ISVs by *in situ* hybridization at 30 hpf (Figure 25A,C), consistent with previous findings [189,190]. Expression analysis by *in situ* hybridization becomes technically challenging or even impossible in embryos older than 2 dpf. Therefore, to analyze expression in ISVs at later stages, when LISV-PLs migrate along aISVs (from 2.5 dpf onwards), we used the *Tp1bglob:eGFP*x*Fli1:DsRed* line [*], in which all endothelial cells are red, and cells with canonical Notch activity are green (GFP expression is driven by a promotor containing 12 Su(H) DNA binding sequences [247]). Imaging of this line at the time when PL cells make the turn and switch from tangential to radial ventral migration revealed that the DA and aISVs are yellow in the merged image, indicating that canonical Notch signaling was active in arterial vessels, but not in LISVs or vISVs (Figure 25F-F"').

Finally, RT-PCR analysis on sorted BEC and LEC fractions after LEC labeling of 4-week old *Fli1:eGFP*^{y1} embryos (see above), confirmed low, but detectable expression levels of Notch-1b in lymphatic structures of older zebrafish (copies Notch-1b/10^5 copies β-actin: 6.9 ± 0.77, *N*=4).

[*] Imaging of this line was performed by Karlien Hermans (Vesaius Research Center, Leuven) in the Lawson lab (Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester)

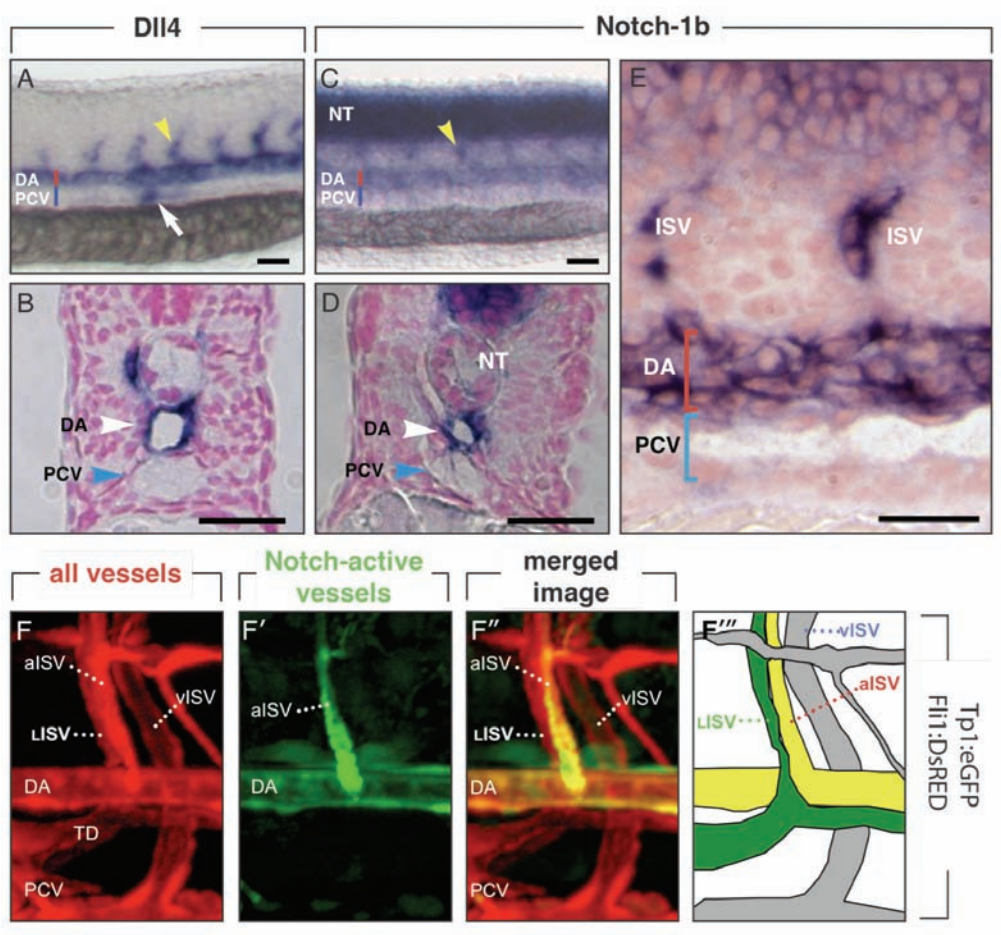

Figure 25: Expression of Dll4 and Notch-1b in zebrafish. A-D, *Whole-mount embryos at 30 hpf, when lymphangiogenic sprouting occurs, were stained for Dll4 (A,B) or Notch-1b (C,D); panels B,D show cross-sections of the respective embryos. Primary ISVs are indicated by yellow arrowheads (A,C).* ***A,B,*** *Dll4 expression was detected in the DA and primary aISVs, besides its known expression in the pronephric duct (white arrow in A).* ***C,D,*** *Notch-1b is strongly expressed in the neural tube (NT), and in the DA and primary aISVs.* ***E,*** *Sagital section of an embryo at 30 hpf, whole-mount stained for Notch-1b. Notch-1b is expressed in the DA and ISVs. A weak signal can be observed in dispersed cells at the dorsal side of the PCV.* ***F,*** *Confocal images of the trunk vasculature in untreated* Tp1bglob:eGFP*x*Fli1:DsRed *embryos, in which* Fli1:DsRed *marks blood and lymph vessels in red (F) and* Tp1bglob:eGFP *labels cells with activated canonical Notch activity in green (F'). The merged image shows arterial vessels (DA; aISV) with active Notch in yellow (green-red), while the LISV are only red (F"). In the schematic representation, the lymphatic structures are indicated in green, Notch-activated vessels in yellow, and other vessels in grey (F"'). Representative images of the arterial activation of Notch in a 6-dpf embryo are shown (for technical reasons), but similar data were obtained at 60 hpf. Scale bars: 50 μm.*

Chapter V

DISCUSSION

The cardinal finding of this study is that incomplete silencing or pharmacological inhibition of Notch signaling impairs lymphatic development in zebrafish and tadpoles. Mechanistic analysis in zebrafish indicates that Notch signaling regulates lymphatic development at two distinct steps, initially during the formation of lymphangiogenic sprouts and their descendent PL cells, which give rise to the TD (Figure 26A,B). At a later stage, Notch signaling is required for guided migration of LISV-PLs along aISVs (Figure 26C-C"').

In the following sections the novel findings that were generated during this PhD project are discussed in light of the existing literature. Our data clearly indicate that blocking Notch signaling in zebrafish abolishes lymphatic development at two stages. However, several unresolved issues require further study in order to unravel the exact mechanisms of how Notch signaling mediates its double role during lymphatic development in zebrafish, as further discussed below. Also, the current limitations of the main model that was used for this study, the zebrafish, are discussed, addressing potential further optimizations to render it even more powerful as a lymphatic model.

1 ZEBRAFISH AS A MODEL TO STUDY LYMPHANGIOGENESIS

This study was largely based on results obtained in the zebrafish model. The lymphatic system in zebrafish has only been discovered a few years ago [6,7] and the lymphatic developmental process in zebrafish embryos has not been characterized in detail. It remains to be determined if molecular regulation of lymphangiogenesis in zebrafish is similar to the mammalian situation and if the same key regulators are involved. We have performed a limited study on the role of the mammalian lymphatic competence regulator Sox18 [50] in zebrafish. Its expression in the vasculature appears to be similar to mouse during early stages of vascular development [50,253,262,280]. At the time of lymphangiogenic sprouting from the PCV, Sox18 is expressed in the PCV. However, it is not clear whether Sox18 is also expressed in the lymphangiogenic secondary sprouts and later in the PL string. Our preliminary data of Sox18 morpholino knockdown did not show a clear lymphatic defect. Secondly, we investigated the involvement of Prox1, the key lymphatic specification

regulator described in mammals [40,46-48]. While Prox1 expression in early lymphangiogenic and lymphatic structures in zebrafish was reported previously [7], we nor several specialized collaborators with extensive expertise in zebrafish and/or Prox1 biology, were able to reproduce the previously described lymphangiogenic/lymphatic expression patterns. We have also analyzed the expression of the Prox1 homologues Prox1-like and Prox2, but found no expression in lymphatic structures. Remarkably, at later stages, when the lymphatic vessels have been properly established and are functional, expression of Prox1 could be detected in sorted LECs. We also analyzed the effect of Prox1 inhibition in zebrafish and observed with one Prox1-targeting morpholino impairment of both PL string and TD formation. However, these phenotypic defects were not reproduced by two other independent morpholinos. Taken together, these data are somewhat puzzling and it remains to be elucidated whether mice and fish really differ in their molecular process regulating lymphangiogenesis. If differences exist, they are only partial, since knockdown or inhibition of other known lymhangiogenic factors, including *Ccbe1* [90,91], *Vegf-c* [6,92], *Vegfr-3* [6,92], and *Synectin* ([93] manuscript under revision), cause impaired lymphangiogenesis, highlighting the conservation of several lymphatic regulators between zebrafish and mammals. With respect to Sox18 and Prox1, it is possible that due to the historic additional genome duplication in teleosts [284], other as yet unidentified paralogues for Sox18 and Prox1 may be active and play a redundant role.

The problems encountered during the expression analyzes also point to an important obstacle that renders the use of the zebrafish model to study developmental processes ongoing beyond 2 dpf quite challenging. From this stage onwards, whole mount *in situ* staining in the trunk becomes difficult or even impossible. It appears that beyond a certain stage, the structure of the skin becomes impermeable for the RNA probes. Therefore, new techniques should be developed to overcome this hurdle. Also immunostaining remains difficult in zebrafish, since in most cases antibodies are developed against a mammalian antigen and often do not recognize the corresponding zebrafish protein. However, as a result of ZFIN society efforts raising this issue, two antibody companies (AnaSpec, Fremont, California; Abcam, Cambridge, Massachusetts) have recently announced to start the production of several zebrafish-specific antibodies. An alternative strategy to analyze lymphatic gene expression but to avoid difficult staining procedures would be the creation of

reporter lines expressing a fluorescent marker, regulated by e.g. the Sox18 or Prox1 promoter. However, identification of the functional promoter elements remains challenging. This is especially the case for Prox1, where even for the mouse, none of the promoter sequences tested so far by us and others (personal communications) in reporter transgenes yielded LEC-specific expression. A large-scale promoter or gene trapping screening [285] could be performed to identify specific lymphatic marker genes in zebrafish. This could also result in the establishment of lymphatic-specific reporter lines that would further advance the zebrafish as a model to study the lymphatic developmental process.

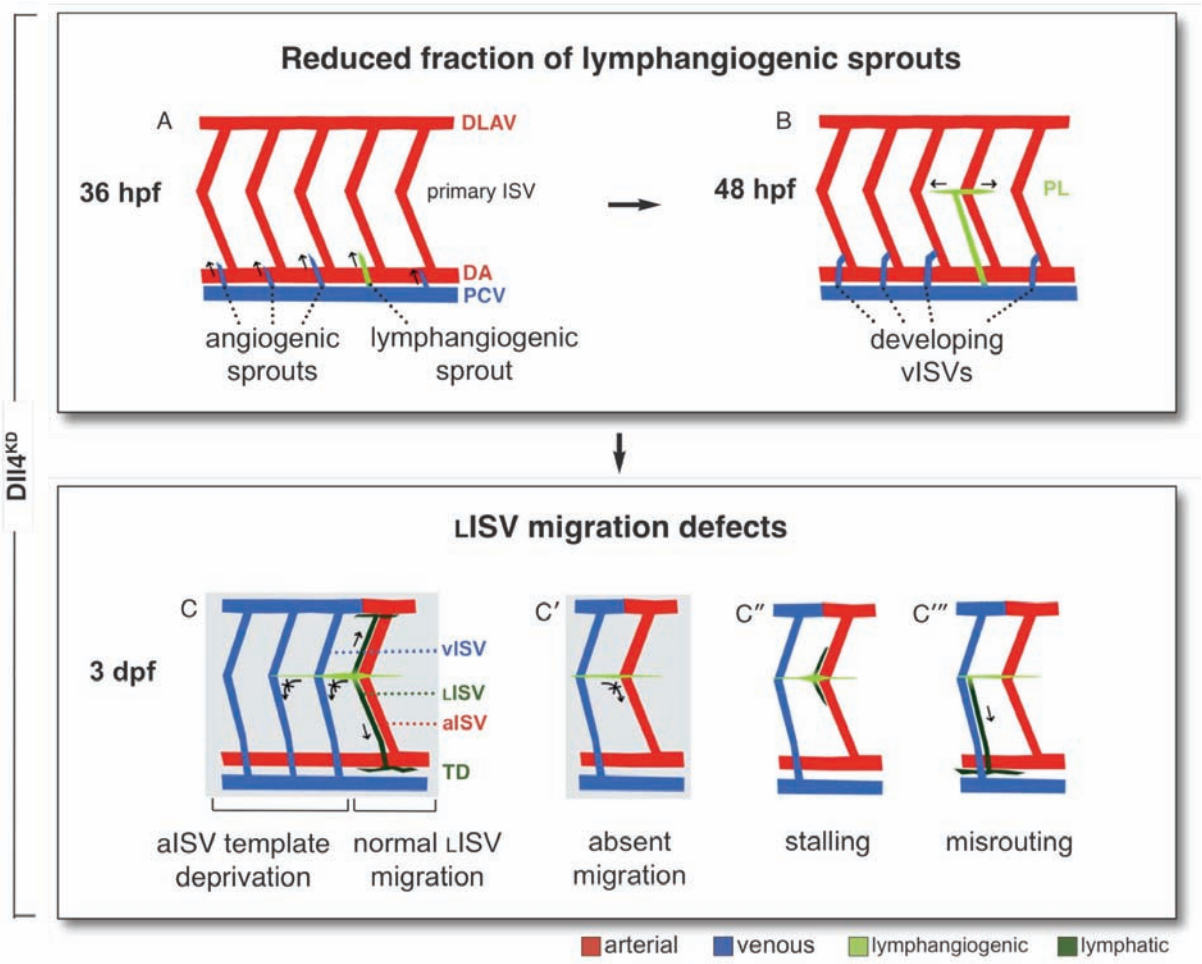

Figure 26: Schematic model of Notch in lymphatic development *Scheme, illustrating the different types of lymphatic defects in Dll4^{KD} embryos (for normal lymphatic development in control embryos, see Figure 3). Permanent lymphatic structures (LISV, TD) are labeled dark green; transient lymphangiogenic structures (lymphangiogenic secondary sprouts; parachordal lymphangioblasts) are labeled light green.* ***A,B,*** *REDUCED FRACTION OF LYMPHANGIOGENIC SPROUTS, resulting in underdevelopment or absence of the PL string, with accompanying overrepresentation of angiogenic secondary sprouts.* ***C-C‴,*** *LISV MIGRATION DEFECTS:* ***C,*** *As a result of the overrepresentation of vISVs, LISV-PLs are deprived of their normal aISV guidance template.* ***C'-C‴,*** *LISV formation is further impaired by additional navigation defects, most frequently because LISV-PLs cells bypass their turning point and never initiate ventral radial migration (C'), or occasionally make the turn but then stall (C''). More rarely, navigating LISV-PLs become misrouted along vISVs (C‴). The most frequent defects are boxed in grey.*

2 ROLE OF NOTCH IN LYMPHANGIOGENIC SECONDARY SPROUT FORMATION

Our results reveal that Notch, in addition to its role in blood vessel morphogenesis and arterial development [180,244], also regulates lymphatic development. Up to half of the Notch hypomorphant embryos failed to form a TD without later rescue, indicating that lymphatic development in the trunk was aborted and not simply delayed. Treatment of zebrafish embryos with DAPT at different stages of lymphatic development revealed that upon early treatment, at stages before or during secondary sprouting from the PCV, most surviving embryos did not form a TD. This suggested an early role for Notch in lymphatic development. DAPT treatment in the tadpole model confirmed the impaired lymphatic development. The earliest identifiable abnormality in Notch-silenced zebrafish embryos, the increased fraction of venous ISVs, reflected a defect at the level of the secondary sprouts from the PCV, where fewer lymphangiogenic but more angiogenic sprouts developed (Figure 26A,B). The lymphangiogenic secondary sprouts give rise to the string of PL cells, which in turn form LECs of the TD. This explains the correlation of the shifted angiogenic/lymphangiogenic balance upon Notch silencing with defective formation of the PL and TD. The reduced fraction of lymphangiogenic sprouts could result from defects in LEC fate acquisition, migration, proliferation, survival and/or other cellular processes contributing to sprout formation and maintenance. How Notch signaling regulates lymphatic development remains unresolved, largely due to technical limitations in the zebrafish model, which have precluded us from determining the exact stages of lymphatic competence, specification and differentiation as defined during mammalian lymphangiogenesis [39]. Therefore, and to avoid any overstatement, the PL-forming secondary sprouts from the PCV, as well as the PL string itself, were termed “lymphangiogenic” to denote their participation in lymphatic development. It is unknown whether ECs of these sprouts merely have the potential to become a LEC (“competence”), or are already programmed to become a LEC, albeit reversibly (“specification”) [39,286]. However, a number of findings suggest that the early lymphangiogenic structures belong to the lymphatic lineage. Indeed, the lymphangiogenic sprouts and PL cells contribute only to lymphangiogenesis but not to angiogenesis, are not labeled in the *kdr-l:mCherryRed* line which marks only arterial and venous ECs [90], and fail to form upon silencing of genes that regulate lymphangiogenesis in mice and humans, e.g.,

Ccbe1 [90,91], *Vegf-c* [6,90], *Vegfr-3* [6,92], *Synectin* ([93], manuscript under revision) and *Prox-1* (unpublished, this thesis). Although our findings that Notch upregulates LEC-specific markers in venous ECs *in vitro* could suggest a role for Notch in the acquisition of lymphangiogenic / lymphatic properties, other mechanisms remain possible.

Based on the present and recent other studies, three possible (non-exclusive) models can be considered to explain our findings. A first explanation is that Notch silencing altered blood vessel development and, secondarily, influenced lymphatic development. Previous studies documented that arterial differentiation is impaired by inhibition of multiple Notch signaling pathways (for instance by a dominant negative Su(H)) [172], but not by selective silencing of Dll4 [189,190]. Our imaging and marker expression analyses are consistent with these findings and reveal that initial formation and differentiation of the PCV, DA and primary ISVs all occurred normally upon incomplete silencing of Notch signaling. Thus, at least by generally accepted criteria of arterial and venous identity, these blood vessels developed normally in Dll4^{KD} and Notch-1b^{SPL} embryos. Nonetheless, we do not exclude the possibility that subtle alterations in arterial characteristics of the primary ISVs might have favored supernumerary connections with secondary sprouts, thereby "entrapping" sprouts that would otherwise have remained lymphangiogenic. Also, Notch silencing resulted in a greater fraction of venous than arterial ISVs; since arterial ISVs act as guidance templates for LISVS, impaired migration of the latter was indeed attributable to such a change in arterial morphogenesis. However, an outstanding question is whether the aISV changes themselves were in fact not caused by defective formation of the lymphangiogenic sprouts in the first instance (reflected in model 2 and 3). Indeed, precisely because lymphangiogenic branches failed to develop in Notch morphants, venous angiogenic sprouts formed instead, which then connected to the primary ISVs and converted them to vISVs.

A second model is that Dll4 and Notch are expressed by the same or adjacent arterial ECs within the DA and that this *cis* signaling induces the release of paracrine lymphangiogenic factors (such as EphrinB2, VEGF-D [10,287] or an unknown signal), that indirectly instruct venous ECs of the nearby PCV to induce lymphangiogenic sprout formation in a cell non-autonomous manner. A similar indirect model was proposed to explain segregation of the DA from PCV in zebrafish [177]. Likewise, during LISV migration,

release of a guidance signal from aISVs in response to Dll4/Notch signaling in arterial cells could assist navigation of LISVs to their target projection (see further).

Finally, a third and perhaps the most appealing, but at this stage still speculative, explanation for our data is that arterial Dll4 in the DA signals *in trans* to Notch on ECs in the PCV, which lies in close juxtaposition at the time of lymphangiogenic sprouting. However, there are arguments in support and against this model. An argument in favour for a cell autonomous role of Notch in PCV cells is that activation of Notch by Dll4 upregulated several LEC-specific markers in venous ECs *in vitro*. Expression analysis experiments *in vivo* yielded indecisive results. Notch-1b expression was weakly detectable in dispersed dorsal PCV cells, but only at a very low level that approached the detection limit of the techniques used. Notch-1b was also measurable by RT-PCR in isolated LECs in older embryos, but this technique could not be used during early lymphatic development. We therefore acknowledge that the Notch-1b expression results represent a limitation of this study, which precludes us from drawing firm conclusions regarding a cell-autonomous role for Notch in lymphangiogenic sprouting.

Another very recent study also documented a cell autonomous role for Notch [237], while a second did not [235]. In LEC cultures, Notch signaling reprogrammed lymphatic to arterial cell fate, while Prox1 counteracted this force, thereby allowing fine-tuning of the LEC fate in a delicately balanced feedback [237]. These findings should not necessarily be in contradiction to our findings, as these researchers analyzed reprogramming of fully differentiated LECs away from their lymphatic fate, while we used venous BECs to study programming towards the LEC fate *in vitro*. As the authors of this study mention [237], “LEC-fate may not be governed by a two-way turn ON-OFF switch, but rather by a dial switch that allows a *gradient* increase or decrease in the lymphatic cell fate force”. Reconciling these and our findings, it seems that Notch levels must be tightly controlled to induce and maintain LEC fate. Low levels of Notch signaling might be required to induce lymphatic fate in venous BECs and, once differentiated into LECs, Prox1 would then secure lymphatic fate by preventing overexpression of Notch, as this would promote arterial cell fate [237]. The lower expression of Notch-1 in LECs (this study and [177,237,240,243,244]) than in arterial ECs supports this model and could also explain why incomplete Notch silencing sufficed to abrogate lymphatic but not arterial development. However, in the absence of more conclusive evidence that Notch

silencing abrogates Prox1 induction in PCV cells in the zebrafish model *in vivo*, a role for Notch in programming LEC fate remains unproven. Also, Notch may regulate other processes than LEC specification in lymphangiogenic sprouting.

A recent study in mice further adds complexity to this model. Indeed, conditional inactivation of RbpJ, a mediator of canonical Notch signaling, in ECs did not alter the expression of lymphatic markers in venous ECs [235]. While these data may suggest that Notch signaling is redundant for LEC specification in mammals *in vivo*, an alternative interpretation is that Notch regulates this process via non-canonical or even reverse signaling. This might also explain why we could not detect a robust signal in LECs or their precursors in the *Tp1bglob:eGFP*x*Fli1:DsRed* line. Unfortunately, in zebrafish, knockdown of Su(H), the homologue of RpbJ, induced too severe developmental defects to analyze its involvement in lymphatic development. Also, species-specific differences between mammals and zebrafish could account for some of the observations. Overall, whether Notch signaling regulates lymphatic development in a cell-autonomous manner remains to be further elucidated in the future.

Since Prox1 has been identified as the key regulator of lymphatic specification an important aspect of further research is to determine if Notch signaling is acting up- or downstream of or independent and in parallel with Prox1. In mice, Prox1 expression is induced at E9.5 in a subpopulation of venous ECs indicating lymphatic commitment of these cells [288]. The factor inducing the polarized expression of Prox1 remains to be identified and also, the subsequent process of budding and migration of LECs is only partially understood. Our preliminary findings point to a possible synergistic inhibitory effect on lymphatic development upon knockdown of Prox1 and Dll4. Once the exact role of Prox1 during lymphangiogenesis in zebrafish has been confirmed (see higher), rescue experiments should clarify the order and hierarchy of the different lymphatic regulatory processes.

Another important transcription factor that possibly links Notch signaling to lymphatic specification but for which the exact lymphangiogenic role remains to be further unraveled, is COUP-TFII. This transcriptional regulator is known to be essential for venous specification by blocking Notch-regulated arterial differentiation [179]. Recent publications have pointed to an important role for COUP-TFII during lymphatic development as well [233-237]. Expression

was shown in lymphatics and COUP-TFII was found to physically and functionally interact with the Prox1 transcription factor [233,234]. Furthermore, deletion in mice impairs development of lymphatic vasculature [235,236]. Considering the venous role of COUP-TFII to block Notch signaling, these findings suggest that Notch signaling needs to be inactivated for lymphatic specification as well. However, this contradicts our findings which document reduced lymphatic development upon Notch inhibition. Another proposed hypothesis is that COUP-TFII indicates the prerequisite of a venous identity for lymphatic specification and that it differentially functions in venous and lymphatic ECs by interacting with other transcriptional regulators [289]. In that regard, it is striking that COUP-TFII is able to negatively and positively regulate Prox1-induced VEGFR3 expression in blood and lymphatic ECs, respectively [234], whereas another study shows that Prox1 and COUP-TFII can either co- or counter-activate expression of different target genes [233]. Altogether, these findings indicate that interaction of Prox1 and COUP-TFII can modulate each other's function depending on the cellular context and other transcriptional regulators. Merely speculating, it is thus possible that COUP-TFII initially blocks Notch signaling in order to obtain a venous identity, after which, in a subset of venous ECs, interaction with Prox1 abolishes the inhibitory effect of COUP-TFII and re-enables Notch signaling to induce lymphatic specification.

3 ROLE OF NOTCH IN LYMPHATIC MIGRATION FROM THE PL

Notch signaling also regulated the formation of LISVs, which arise from the PL cells. We have observed that LISV-PLs migrate exclusively along arterial ISVs, indicating that the PL cells must recognize a turning signal at the crossing with an aISV that instructs them to switch from tangential to radial migration. As indicated above, due to the shifted balance of angiogenic/lymphangiogenic secondary sprout formation Notch hypomorphants have a reduced availability of aISV templates, thus largely depriving PL cells from normal migration routes and thereby impairing normal TD formation. However, even though the arterial-venous ISV shift was most prominent in hypomorphant embryos with the most severe lymphatic defects, not all aISVs underwent a venous shift (maximally ≈90%), possibly reflecting an incomplete knockdown or, perhaps, a fundamental physiological need to

establish at least some residual trunk circulation from aISVs to vISVs. When analyzing Notch hypomorphants with a partial or normal PL string, we observed that in the remaining fraction of aISV, most frequently, the LISV was absent and in other, rarer cases, migrating LISV-PLs stalled or became misrouted (Figure 26C-C'''). Our findings suggest that lymphangiogenic EC migration *per se* (motility) was normal. Indeed, in embryos that formed a partial TD, cells of the lymphangiogenic secondary sprouts migrated dorsally without stalling and thereafter tangentially to establish the string of PL cells. Also, for PL cells that finished their ventral migration to the TD, Notch signaling was dispensable for their subsequent tangential outgrowth (not shown). Furthermore, *in vitro* migration or proliferation of LECs was also not affected by Notch signaling. Also, we did not detect signs of lymphatic regression or retraction (not shown). It is therefore tempting to speculate that LISV defects in Notch-silenced embryos reflect impaired lymphangiogenic cell pathfinding. LISV-PLs navigated in close association along aISV templates, raising the question whether aISVs act as guidance templates for LISV-PLs, reminiscent of how *follower* axons navigate along a *pioneer* axon's pathway or how autonomic nerves use arterial tracks to reach their target [290,291]. Hence, it is tempting to speculate that, when fewer aISVs are present in Notch hypomorphants because of lymphangiogenic sprouting defects, PL cells are deprived of navigation templates and therefore cannot form LISVs normally (Figure 26C). The observations that LISV-PLs failed to switch from tangential to radial migration or, more rarely, stalled or selected incorrect paths (Figure 26C'-C'''), are reminiscent of classic neuronal guidance defects. That arteries may act as navigation templates is evidenced by reports that autonomic nerves stall or become misrouted, when these arteries do not produce appropriate guidance cues [291]. Su(H)-dependent Notch activity was detectable in aISVs, but not in vISVs, at the time when PL cells switch from tangential to radial migration alongside aISV, indicating that lymphatic navigation is regulated either non-cell autonomously or via non-canonical Notch signaling. Considering the close association of LISVs and aISVs, Notch signaling in the latter could indirectly signal to the former and thereby regulate lymphatic development.

Whether and how Notch signaling regulates the production of turning and guidance cues for LISV-PL cells by aISVs or nearby (somitic) cells and how LECs recognize these signals remains to be determined. It would be interesting to analyze the specific behavior of

lymphatic tip cells during normal LISV pathfinding and to examine if inhibition of Notch signaling has similar effects in arterial and lymphatic sprouting. Notch has previously been implicated in similar processes of neuronal migration and axon guidance in *Drosophila*. In this context, Notch acts via a non-canonical CSL-independent manner by direct activation of the Abl tyrosine kinase by Notch receptors [292,293]. This process involves a range of other proteins, such as Disabled, of which the mouse homolog Disabled1 was also shown to interact with the intracellular domain of Notch1 [292-294]. It has been speculated that a similar CSL-independent pathway might be involved in regulating EC migration downstream of Notch [245]. Similarly, our observations of impaired LISV navigation upon Notch inhibition and absence of canonical Notch activation in the LISV, could be in agreement with such a regulatory process during lymphatic pathfinding.

Notch1 and Notch4 have been shown to be expressed in normal and pathological lymphatics in mouse and human [230] and cultured LECs express some of the Notch receptors (Notch-1, -2 and -4), ligands (Dll1, Jagged1, Jagged2) and downstream targets (Hey1 or -2) [231,232]. However, no lymphatic abnormalities have been described in Notch mutant mice, possibly because these mutants die earlier than the onset of lymphatic vessel development due to vascular malformations [295]. It therefore remains to be determined whether conditional inactivation of the other Notch pathway components at later stages, after critical vascular developmental steps and before lymphatic specification, unlike knockout of RbpJ [235], do reveal a role for Notch signaling during the different steps of mammalian lymphangiogenesis.

4 GENERAL CONCLUSION AND PERSPECTIVES

In conclusion, this study provides novel insight for a role of Notch signaling in lymphatic development, in part by regulating the initial steps of lymphangiogenic sprouting and PL formation. The findings raise the interesting perspective that Notch regulates, directly or indirectly, the potential of venous ECs to become lymphangiogenic / lymphatic. Moreover,

the navigation defects of LISV-PL cells along aISVs suggest that Notch also regulates lymph vessel pathfinding along arteries.

Further study is required in order to unravel the exact molecular mechanisms in which Notch signaling is involved to regulate lymphatic development. As addressed during the discussion above, the key questions to further unravel include:

- Is the observed Prox1/Dll4 synergy in zebrafish embryos reflecting interaction between the Prox1 and the Notch pathway in lymphangiogenesis and how do they chronologically and hierarchically relate to one another?
- Is Notch signaling involved in the regulation of LEC competence/commitment, in LEC maintenance, LEC migration, or possibly in several of these steps?
- Does Notch signaling from the DA to the PCV occur directly or indirectly to regulate secondary lymphangiogenic sprouting? Or is the reduced number of lymphangiogenic sprouts an indirect result of vascular or other abnormalities?
- Does Notch signaling regulate PL cell migration along aISVs in zebrafish embryos in a cell-autonomous or cell non-autonomous manner? Which are the guidance cues and responsive molecules that instruct PL cells to switch from tangential migration at the horizontal myoseptum to radial migration along the aISVs?
- Is Notch regulation of lymphangiogenesis different in zebrafish and mammals? Do conditional knockouts of specific Notch pathway components in mice (as opposed to the general knockout of canonical Notch signaling in RBPj knockouts [235]) display lymphatic abnormalities?

On a more general level, further characterization of the consecutive stages of lymphangiogenesis in zebrafish embryos is required as well:

- What is the molecular mechanism underlying LEC specification? Are Sox18 and Prox1 involved in zebrafish lymphangiogenesis as in mammals?
- Is there a genetic difference between the angiogenic and lymphangiogenic secondary sprouts of the PCV? etc.

Elucidation of these issues together with development of improved tools and methods to specifically stain lymphangiogenic/lymphatic structures at different stages of lymphangiogenesis, will greatly advance lymphangiogenesis research in the zebrafish model in general, as well as be instrumental to further unravel in-depth the precise involvements of the Notch signaling pathway.

Chapter VI

SUMMARY
SAMENVATTING

1 SUMMARY

Despite the importance of lymph vessels in both health and disease, very little is known about the genetic regulation of lymphangiogenesis, the formation of lymph vessels. The Notch signaling pathway is involved in many developmental processes. During angiogenesis, the formation of blood vessels, Notch regulates arterial *versus* venous differentiation of blood endothelial cells and controls the branching process of the blood vessel network. The molecular mechanisms determining the establishment of lymphatic endothelial cells, the third type of endothelial cells, and the outgrowth of an elaborate network of lymph vessels, are still poorly understood. Several indications suggest a role for Notch in all three EC types. Components of the Notch pathway are expressed in LECs and several Notch-related genes that are important for arterial differentiation are also required in lymphatic development. We therefore reasoned that Notch signaling itself might also be involved in lymphangiogenesis. In this study we investigated the role of the Notch signaling pathway in lymphangiogenesis using zebrafish embryos and tadpoles, two animal models that allow high-throughput screening for lymphatic genes. By adapting chemical and genetic silencing strategies to avoid vascular defects, we identified for the first time a dual role for Notch signaling in developmental lymphangiogenesis.

We first showed that general inhibition of Notch signaling by a gamma-secretase inhibitor in zebrafish embryos and tadpoles or by knockdown in zebrafish of presenilin-1, involved in Notch activation, blocks lymphatic development.

We next investigated further in the zebrafish model which of the known Notch receptors and ligands were the main actors and identified crucial roles of the Notch ligand Dll4 and the zNotch-1b/6 receptors, knockdown of which all arrest lymphatic development.

In addition, we examined which developmental steps during lymphangiogenesis were affected, uncovering a dual action of Dll4/Notch signaling. During early stages, Dll4/Notch silencing reduces the number of sprouts that give rise to the parachordal lymphangioblasts (lymphangiogenic sprouts) from their venous precursor; instead, angiogenic sprouts are formed. This directly results in an increased formation of venous connected intersomitic vessels and impaired development of the parachordal lymphangioblast string due to a reduced delivery of parachordal lymphangioblasts at the horizontal myoseptum. At a later

phase, Notch silencing impairs navigation of lymphatic intersomitic vessels along their arterial templates. Together, the early and later defects result in impaired formation of the thoracic duct, the first functional lymph vessel in the zebrafish trunk.

These findings imply critical roles for Notch signaling in the formation and wiring of the lymphatic network and raise the intriguing questions whether lymphatic and arterial endothelial cells coopted similar classes of signals from each other to acquire a non-venous EC identity and whether Notch signaling regulates their close physical association. The exact molecular mechanisms by which Notch signaling regulates both steps of the lymphangiogenic process remain to be defined.

2 SAMENVATTING

Ondanks het belang van lymfevaten zowel voor het normaal functioneren van het lichaam als voor hun betrokkenheid in verschillende ziektebeelden, is er slechts weinig geweten over de genetische regulatie van lymfangiogenese, de ontwikkeling van lymfevaten. De Notch signaalweg is betrokken in vele ontwikkelingsprocessen. Tijdens angiogenese, de vorming van bloedvaten, zorgt Notch voor de differentiatie van arteriële *versus* veneuze bloedvat endotheelcellen, en controleert het de vertakking van het bloedvatennetwerk. De moleculaire mechanismen die het ontstaan regelen van lymfatische endotheelcellen, de derde soort endotheelcellen, en de vorming van een uitgebreid net van lymfevaten, zijn veel minder goed gekend. Er zijn meerdere indicaties dat Notch een rol zou kunnen spelen in alle drie de endotheelcel types. Componenten van de Notch signaalweg komen tot expressie in lymfatische endotheelcellen en verschillende Notch-gerelateerde genen zijn zowel belangrijk voor arteriële differentiatie als voor lymfatische ontwikkeling. Dit leidde ons tot de hypothese dat ook de Notch signaalweg zelf belangrijk zou kunnen zijn voor lymfatische ontwikkeling. Tijdens dit onderzoek hebben we de betrokkenheid van de Notch signaalweg bestudeerd tijdens lymfangiogenese door gebruik te maken van de zebravis en de kikkervis, twee diermodellen die kunnen gebruikt worden voor 'high-throughput' identificatie van lymfatische genen. Door gebruik te maken van farmacologische en genetische inhibitie strategieën en de inhibite zo te doseren dat bloedvatdefecten worden vermeden, zijn we erin geslaagd om voor de eerste keer een dubbele rol aan te tonen voor Notch tijdens lymfangiogenese.

In een eerste reeks experimenten, toonden we aan dat algemene inhibitie van Notch signalering met behulp van een gamma-secretase inhibitor in zebravis embryo's en kikkervisjes, of specifieke inhibitie in zebravis embryo's van preseniline-1 (via morpholino knockdown), lymfatische ontwikkeling blokkeert.

Vervolgens onderzochten we in het zebravismodel welke van de gekende Notch liganden en receptors de belangrijkste actoren zijn, en identificeerden een cruciale rol voor de Notch ligand Dll4 en de receptors Notch-1b en -6. Specifieke morpholino knockdown van deze signaalwegcomponenten resulteerde telkens in verstoorde lymfatische ontwikkeling.

Tenslotte gingen we na welke stappen tijdens lymfangiogenese getroffen waren, waarbij Dll4/Notch signalering een tweevoudige rol bleek te hebben. Tijdens vroege stadia leidt inhibitie van Dll4/Notch tot een kleiner aantal lymfangiogene vertakkingen die afsplitsen van de vene; in plaats daarvan wordt een groter aantal angiogene vertakkingen gevormd. Deze afwijking van de normale ~50:50 verhouding, resulteert rechtstreeks in een verhoogde vorming van veneus geconnecteerde intersomietische bloedvaten en een verstoorde ontwikkeling van de parachordale lymphangioblast koord door een verminderde aflevering van parchordale lymphangioblasten ter hoogte van het horizontaal myoseptum. Tijdens een latere fase verstoort inhibitie van Notch de navigatie van lymfatische intersomitische vaten langsheen hun arteriële migratieweg. Deze vroege en latere defecten resulteren in verstoorde vorming van de thoracale buis, het eerste functionele lymfevat in het lichaam van het zebravis embryo.

Deze bevindingen tonen aan dat Notch signalering cruciaal is tijdens de vorming en uitgroei van het lymfatisch netwerk en roept de intrigerende vragen op of lymfatische en arteriële endotheelcellen gelijkaardige signalen gebruiken om een niet-veneuze identiteit te bekomen, en of Notch signalering het nauwe fysische contact dat waargenomen is tussen arteriële en lymfatische vaten, regelt. Verder onderzoek is vereist om de exacte moleculaire mechanismen te ontrafelen die Notch gebruikt om beide stappen van het lymfangiogenese proces te reguleren.

Chapter VII

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PUBLICATION LIST

- Ny A, Koch M, Vandevelde W, Schneider M, Fischer C, Diez-Juan A, Lambrechts D, Neven E, **Geudens I**, Maity S, Plaisance S, Lambrechts D, Carmeliet P and M Dewerchin. Role of VEGF-D and VEGFR-3 in developmental lymphangiogenesis, a chemicogenetic study in Xenopus tadpoles. *Blood 112(5),1740-1749, 2008*.
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INTERNATIONAL MEETINGS

- March 2-7 2008: Gordon Research Conference, Molecular Mechanisms In Lymphatic Function & Disease, Ventura, CA. *Poster Presentation*: “Dll4/Notch signaling plays a crucial role during lymphatic development in zebrafish embryos and *Xenopus laevis* tadpoles”
- June 4-5 2009: EMBO Workshop, Lymphatic and Blood Vasculature: From models to human disease, Helsinki, Finland. *Poster Presentation*: “Role of Dll4/Notch signalling in lymphatic development”
- June 6-7 2009: European School of Haematology, Interdisciplinary Conference on Angiogenesis, Helsinki, Finland. *Poster Presentation*: “Role of Dll4/Notch signalling in lymphatic development”